53. (Amended) A viral vector comprising a nucleic acid of claim 1.

a5

54. (Amended) A fusion polypeptide comprising an amino acid sequence encoded by the nucleic acid sequence of claim 1 fused to a heterologous amino acid sequence.

a6

70. (Amended) A nucleic acid according to claim 1 attached to a solid support.

a7

72. (New) The process of claim 8 further comprising isolating the polypeptide from the culture.

REMARKS

I. Explanation of Amendments

A marked-up version of the changes made to the claims and specification can be found in Appendix A hereto. As a convenience to the examiner, the applicants have set forth the pending claims in Appendix B as they should appear after entry of the foregoing amendment.

In paragraph 2 of the Office action, the examiner objected to the title of the invention. Specifically, the examiner alleged the title was not descriptive. Applicants have amended the title and are now clearly indicative of the invention to which the claimed are directed.

In paragraph 3 of the Office action, the examiner objected to claims 1-8, 10, 11, 51-55, 70, and 71 for reciting non-elected sequences SEQ ID NO: 3 and SEQ ID NO: 4. Amendments removing the recitation to the non-elected SEQ ID NOS: 3 and 4 in claims 1, 4, 51, 53, and 70, and the cancellation of claims 2, 3, and 71 obviate this objection.

In paragraph 3 of the Office action, the examiner also objected to claims 54 and 55 for reciting non-elected claims 13, 14, and 15. The applicants have amended claims 54 and 55 to address the objection. Support for the nucleic acids recited in claims 54 and 55 can be found in the claims as originally filed and in the specification, from page 35, line 11, to page 38, line 10.

Amended claims 1, 4, 51, 52, and 53 have been amended to remove the unnecessary word "molecule", an amendment which is not narrowing and which is not related to

patentability. Claim 1 was further amended in part (b) to recite a nucleotide sequence encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2.

Amended claim 8 recites a process of producing a CD20/IgE-receptor like polypeptide comprising culturing the host cell of claim 5 under suitable conditions to express a CD20/IgE-receptor like polypeptide encoded by the nucleic acid. This amendment finds support in the parent claims from which claim 8 depends, and is not in response to any rejection.

Amended claim 10 recites a process of claim 8 wherein the vector further comprises a heterologous promoter operatively linked to the nucleotide sequence encoding the CD20/IgE-receptor like polypeptide. This amendment states the subject matter of the claim more succinctly without narrowing the scope of the claim. Support for the phrase "heterologous promoter" is found throughout the specification, for example, on page 52, lines 2-9.

Claim 70 has been amended to recite a term with verbatim antecedent basis and not to narrow the claim or for any reason related to patentability.

New claim 72 recites the process of claim 8 further comprising isolating the polypeptide from the culture. Support for the isolation of the polypeptide can be found in originally filed claim 8 and in the specification on pages 58 and 59.

Claims 9, 12-50, and 56-69 were cancelled because these claims were withdrawn from consideration as being drawn to a non-elected invention, and not for any reason related to patentability. The applicants do not intend by these or any amendments to abandon subject matter of the claims as originally filed or later presented, and reserve the right to pursue such subject matter in continuing applications.

II. The Rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

A. The Claims Are Directed to Statutory Subject Matter

In paragraph 4 of the Office action, the Patent Office rejected claims 5-7 under 35 USC § 101 for allegedly being directed to non-statutory subject matter. The examiner asserts that the recited host cells in claims 5-7 encompass human cells, fetuses and embryos as well

as non-human cells including chimeric animals, germ cells, fertilized eggs, fetal tissues and organs.

In its rejection, the Patent Office seems to be stating that subject matter is non-statutory if it can be read to "embrace" a cell in a human or animal. However, no authority is cited for this proposition. In fact, the Patent Office has granted numerous patents on subject matter that is used in humans. For example, pharmaceuticals and foodstuffs are patentable subject matter, even though they are administered to or ingested by humans, or animals. Medical devices, such as artificial body parts, implantable stents and prostheses, and the like, are patentable subject matter, even though they are implanted transiently or permanently in a human or animal. These are just some examples of statutory subject matter that can transiently or permanently be introduced to a human.

The foregoing examples can be distinguished from a patent claim to a human per se. However, there is no basis for interpreting claims 5-7 as reading on a human per se, just as there is no basis for interpreting medical device claims as reading on the patients into whom the device is implanted. For these reasons, the rejection should be withdrawn.

In the Office action, the Patent Office also suggested that amending the claims to recite "non-human" would obviate the rejection. This suggestion has not been adopted because the applicants contemplate human host cells, and there is no statutory prohibition against them. For example, the application describes using human cell lines, such as human embryonic kidney cells (HEK) 293 or 293T cells (page 56, lines 15 and 16) for recombinant production of the polypeptide.

The Patent Office also suggested that amendment to reflect "hand-of-man" would be appropriate. However, claim 5 already requires the "hand-of-man" to make a vector comprising the nucleic acid and to introduce the vector into a host cell, so no further amendment is believed to be necessary.

B. The Claims Are Directed to a Credible, Specific and Substantial Utility

In paragraph 5a of the Office action, the Patent Office rejected claims 1-8, 10, 11, 51-55, 70, and 71 under 35 U.S.C. § 101 for assertedly lacking a credible, specific, and substantial or well-established utility. Specifically, the examiner alleged that the specification does not reasonable provide enablement to obtain the functional data needed to permit one to produce a nucleic acid with the functional (a defined biological activity)

requirements of the claims. Cancellation of claims 2, 3, 11 and 71 by amendment herein has rendered moot the rejections as applied to those claims. The applicants respectfully traverse these rejections as applied to claims 1, 4-8, 10, 51-55, and 70.

The Patent Office's basis for rejection focused on whether or not the application contained a sufficient teaching of protein "activity." Not only is this allegation incorrect, but it is also too narrowly focused, because "protein activity" is not the only specific, substantial, and credible utility for the biological molecules claimed. For example, the polynucleotide SEQ ID NO: 1 can be used as a marker for testicular cells specifically expressing this nucleic acid molecule. The specification contemplates using SEQ ID NO: 1 as a tissue specific probe, e.g., page 41, lines 30-32. In Example 3, at page 112, lines 13-18, the application teaches that the DNA sequence from clone agp-96614-al (SEQ ID NO: 1) was used as a probe for a Northern tissue expression experiment and the results show a specific (predominant) tissue expression from the testes.[1] Using SEQ ID NO:1 as a probe is a **"credible"** utility according to page 5 of the Revised Interim Utility Guidelines Training Materials.[2] In addition, this utility is **"substantial"** because a testes-specific probe, like any tissue specific probe, can be used in a variety of real world contexts to identify the tissue type of cells. For example, a pathologist can use tissue specific probes to identify cell types in a biological sample, which maybe helpful, e.g., to screen for a tumor metastases. Thus, polynucleotides of the invention have a credible, specific and substantial utility as a tissue-specific probe.

By way of analogy, the encoded protein, which is a 4-transmembrane protein expressed on cell surfaces, is useful as a tissue specific marker as well, e.g., using antibodies for detection.

In addition, the polynucleotide SEQ ID NO: 1 is useful to map the chromosomal location of CD20/IgE receptor-like gene. The specification teaches to use SEQ ID NO: 1 as a chromosomal marker for itself and related genes on the chromosome on page 105, lines 28-

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[1] This expression profile was independently confirmed in Hulett *et al.* (*Biochem and Biophys. Research Communications 280*:374-379 (2001)) (*See* Appendix C). Compare page 377, second column and Figure 3 of Hulett *et al.* with Example 3 of the specification.

[2] "A credible utility is assessed from the standpoint of whether a person of ordinary skill in the art would accept that the recited or disclosed invention is currently available for such use. For example, no perpetual motion would be considered to be currently available. However, nucleic acids could be used as probes, chromosome markers, or forensic or diagnostic markers." Page 5, lines 11-18 of the Revised Interim Utility Guidelines Training Materials.

33. The use of SEQ ID NO: 1 as a chromosomal marker is a "credible utility" according to the Revised Interim Utility Guidelines Training Materials on page 5 (see footnote 2). SEQ ID NO: 1 encodes for a cell surface receptor and is the member of the MSA4 family (membrane-spanning 4-domain family, subfamily A) whose members include CD20, FcεRIβ (IgE), and HTm4. [*See* Liang *et al.*, *Genomics 72*:119-127, 120 (2001) (See Appendix D) and specification at page 11, lines 5-9, and Figure 3.] This utility as a chromosomal marker has been confirmed/validated in the literature which has reported that SEQ ID NO: 1 and the related CD20, IgE, and HTm_4 genes are clustered within the same region of chromosome 11 (11q 12-13). (*See* Hulett *et al.*, at page 378). Chromosomal aberrations within this region of chromosome 11 are known to be linked to non-Hodgkin's lymphoma (McLaughlin *et al.*, 1998) and pathogenesis of various allergic diseases (Adra *et al.*, 1999) (See Appendix E). Therefore, SEQ ID NO: 1 has a real world use as a chromosomal marker for chromosome 11q 12-13. Chromosomal markers can also be used as a probe for detecting various forms of aneuploidy, genetic disorders characterized by an abnormal number of chromosomes. Thus, the polynucleotides of the invention also have a credible, specific, and substantial utility as a chromosomal marker.

For reasons set forth above, the nucleic acid sequence of SEQ ID NO: 1 has a credible, specific and substantial utility as a probe and as a chromosomal marker. Accordingly, the applicants respectfully requests that the rejection of the claims under 35 U.S.C. §101 be withdrawn.

III. The Rejections Under 35 U.S.C. §112, First Paragraph, Should Be Withdrawn

A. The Claims Are Enabled by the Specification

In paragraph 5b of the office action, the examiner rejected claims 1-8, 10, 11, 51-55, 70, and 71 under 35 U.S.C. §112, first paragraph, for allegedly lacking a substantial asserted utility, such that the specification allegedly did not enable one of skill in the art to use the claimed invention. As explained in detail in the preceding section, the application teaches one of ordinary skill how to use polynucleotides of the invention as a probe or marker, for example.

The examiner further alleged that the specification does not reasonably provide enablement to obtain the functional and structural data needed to permit one to produce a nucleic acid which meets both the structural (at least 70% identical to the polypeptide of SEQ ID NO: 2) and functional (biological activity) requirements of the claims. These rejections have been rendered moot by this amendment.

The applicants have amended claim 1, which is now directed to (a) polynucleotides comprising the nucleotide sequence of SEQ ID NO: 1, (b) polynucleotides that encode the amino acid sequence of SEQ ID NO: 2, and (c) polynucleotides that are fully complementary to (a) or (b). The specification enables one of skill in the art to make and use the nucleic acid molecule of SEQ ID NO: 1 (See discussion above). Accordingly, claim 1 and each one of claims 4-8, 10, 51-55, 70, and 71, which ultimately depend from claim 1, are enabled.

B. The Claims Are Supported by an Adequate Written Description

The examiner alleged that claims containing the phrases "allelic variant or splice variant," "an isolated nucleic acid encoding a polypeptide that is at least about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the polypeptide of SEQ ID NO: 2," "a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of...., wherein the encoded polypeptide has an activity of the polypeptide as set forth in SEQ ID NO: 2," and "at least one conservative amino acid insertion, substitution, or deletion" failed to meet the written description requirement. [Office Action, paragraphs 5c and 5d].

These rejections are now moot. Solely for the purpose of expediting prosecution, the applicants have removed these phrases from claim 1 and cancelled claims 2 and 3, without prejudice. In light of the foregoing amendments, the applicants request that the rejections under 35 U.S.C. §112, first paragraph, be withdrawn.

IV. The Rejections Under 35 U.S.C. §112, Second Paragraph, Should Be Withdrawn

In paragraph 6 of the Office action, the examiner rejected claims 1-8, 10, 11, 51-55, 70, and 71 under 35 U.S.C. §112, second paragraph, as assertedly containing indefinite terminology.

The examiner alleged that the phrases "an activity of the polypeptide set forth in SEQ ID NO: 2" and "hybridizes under moderately or highly stringent hybridization conditions" in claim 1 were indefinite. Solely for the purpose of expediting prosecution, the applicants have deleted these phrases from the claims. In addition, the examiner further alleged that line 3 of claim 1 is improper because the phrase "the nucleotide sequence" lacks antecedent basis. While the applicants believe the phrase in question is conventional in patent claims and susceptible to only one interpretation, they have amended line 3 to "a nucleotide sequence," since such amendment is not narrowing any way. Accordingly, these rejections are rendered moot.

The rejection of claims 2 and 3 are moot, because the claims have been cancelled.

The examiner rejected claim 8 under §112, second paragraph, as being indefinite for the recitation of the term "optionally" in a final isolation step. The applicants have amended claim 8 by removing this final step, and added new dependent claim 72, which recites the process of claim 8 wherein the polypeptide is isolated. These amendments thereby render moot the basis for rejecting claim 8 and its other dependent claims.

The examiner rejected claims 51 and 53 under §112, second paragraph, as being indefinite and vague for the recitation "of claims 1, 2 or 3." Due to the cancellation of claims 2 and 3, claims 51 and 53 have been amended to depend solely to claim 1, thereby rendering moot the basis for rejecting claims 51 and 53 and its dependent claims. In light of the foregoing amendments, the applicants submit that the rejections of claims 1-8, 10, 11, 51-55, 70, and 71 under 35 U.S.C. §112, second paragraph, for indefiniteness has been overcome and should be withdrawn.

V. The Rejection Under 35 U.S.C. §102(b) Should Be Withdrawn

In paragraph 7 of the Office action, the examiner rejected claims 1-3 alleging that these claims were anticipated under 35 U.S.C. §102(b) by Hillier *et al.*, WashU-NCI Human EST Project (1997) (hereafter Hillier *et al.*).

The rejection under 35 U.S.C. §102(b) based on Hillier *et al.* is now moot. Solely for purposes of expediting prosecution, the applicants have amended claim 1 to distinguish the claimed invention from the disclosure of the cited document and cancelled claims 2 and 3 without prejudice. The amended claims recite sequences which are neither disclosed nor

suggested by the cited document. Therefore, the applicants respectfully request that the rejection under §102(b) be withdrawn.

VI. The Rejections Under 35 U.S.C. §103(a) Should Be Withdrawn

In paragraphs 8a and 8b of the Office action, the examiner rejected claims 4-8, 10, 11, and 51-53 under 35 U.S.C. §103(a) as allegedly being obvious in light of the disclosure of Hillier *et al.*, and rejected claims 54 and 55 as allegedly being obvious in light of Hillier *et al.* in view of Capon *et al.* (U.S. Patent Number 5,116,964) (hereafter Capon *et al.*).

These rejections have been rendered moot by the instant amendment of the relevant claims. Claim 11 has been cancelled by amendments herein. Amended claims 4-8, 10, and 51-53 are now directed to either host cells, polynucleotide compositions, or vectors with (a) the nucleic acid sequence of SEQ ID NO: 1; or (b) a nucleotide sequence encoded by a polypeptide having an amino acid sequence as set forth in SEQ ID NO: 2; or (c) a nucleotide sequence fully complementary to (a) or (b) by virtue of their dependency from claim 1. Hillier *et al.* neither teaches nor suggests the nucleic acid molecule set forth in SEQ ID NO: 1 nor a nucleotide sequence that encodes for the amino acid sequence as set forth in SEQ ID NO: 2. Furthermore, Hillier *et al.* fails to disclose or suggest a sequence sufficient in length to encode applicants' polypeptide (in fact Hillier *et al.* fails to disclose or suggest the existence of any encoded polypeptide). One of skill in the art would not be motivated to express the nucleic acid sequences of claim 1 in a host according to this teaching.

The amendment to claim 1 further renders moot the rejection of claims 54 and 55. As discussed above, Hillier *et al.* fails to teach an encoded protein. Capon *et al.* is cited in this rejection only for its teachings related to fusion proteins encoded by a heterologous nucleic acid constructs. Since Hillier *et al.* is silent with regard to encoded proteins, there would have been no motivation to combine Hillier *et al* with Capon *et al.*

Accordingly, Hillier et al., alone or in combination with Capon et al., neither disclose nor suggest the same DNA within vectors, host cells, DNA compositions, or fusion proteins of dependent claims 4-8, 10, and 51-55. Thus, these references do not render claims 4-8, 10, and 51-55 obvious under 35 U.S.C. §103(a).

SUMMARY

In view of the amendment and remarks made herein, the applicants believe that claims 1, 4-8, 10, 51-55, 70, and 71 are in condition for allowance and request notification of the same.

Respectfully submitted,

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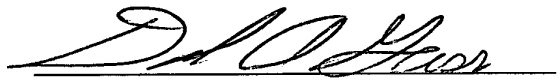
By 

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January 15, 2003

APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Please replace the first line of page 1 with the following title:

Polynucleotides Encoding a CD20/IgE-Receptor Like Molecule and Uses Thereof

IN THE CLAIMS:

Please cancel claims 2, 3, 9, 11-50, 56-69, and 71.

Please amend claims 1, 5-7, 51, 53, 54, and 70.

1. (Amended) An isolated nucleic acid [molecule] comprising a nucleotide sequence selected from the group consisting of:

(a) [the] a nucleotide sequence set forth [in either SEQ ID NO: 1 or SEQ ID NO:3] in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide [having] comprising an amino acid sequence as set forth in [either SEQ ID NO: 2 or SEQ ID NO: 4] SEQ ID NO: 2; and

(c) a nucleotide sequence fully complementary to [any of (a) - (c)] (a) or (b).

4. (Amended) A vector comprising the nucleic acid [molecule] of [claims 1, 2, or 3] claim 1.

8. (Amended) A process of producing a CD20/IgE-receptor like polypeptide comprising culturing the host cell of claim 5 under suitable conditions to express [the polypeptide, and optionally isolating the polypeptide from the culture.] a CD20/IgE-receptor like polypeptide encoded by the nucleic acid.

10. (Amended) The process of claim 8, wherein the [nucleic acid molecule] vector further comprises a heterologous promoter [DNA other than the promoter DNA for the

native CD20/IgE-receptor like polypeptide] operatively linked to the [DNA] nucleotide sequence encoding the CD20/IgE-receptor like polypeptide.

51. (Amended) A composition comprising a nucleic acid [molecule] of [claims 1, 2, or 3] claim 1 and a pharmaceutically acceptable formulating agent.

52. (Amended) A composition of claim 51 wherein said nucleic acid [molecule] is contained in a viral vector.

53. (Amended) A viral vector comprising a nucleic acid [molecule] of [claims 1, 2, or 3] claim 1.

54. (Amended) A fusion polypeptide comprising [the polypeptide of claims 13, 14, or 15] an amino acid sequence encoded by the nucleic acid sequence of claim 1 fused to a heterologous amino acid sequence.

70. (Amended) A [polynucleotide] nucleic acid according to [any one of claims 1 to 3] claim 1 attached to a solid support.

72. (New) The process of claim 8 further comprising isolating the polypeptide from the culture.

APPENDIX B

ELECTED CLAIMS STILL PENDING UPON ENTRY OF THE FOREGOING AMENDMENT

1. (Amended) An isolated nucleic acid comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence set forth in SEQ ID NO: 1;

(b) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO: 2; and

(c) a nucleotide sequence fully complementary to (a) or (b).

4. (Amended) A vector comprising the nucleic acid of claim 1.

5. (Amended) A recombinant host cell comprising the vector of claim 4.

6. (Amended) The host cell of claim 5 that is a eukaryotic cell.

7. (Amended) The host cell of claim 5 that is a prokaryotic cell.

8. (Amended) A process of producing a CD20/IgE-receptor like polypeptide comprising culturing the host cell of claim 5 under suitable conditions to express a CD20/IgE-receptor like polypeptide encoded by the nucleic acid.

10. (Amended) The process of claim 8, wherein the vector further comprises a heterologous promoter operatively linked to the nucleotide sequence encoding the CD20/IgE-receptor like polypeptide.

51. (Amended) A composition comprising a nucleic acid of claim 1 and a pharmaceutically acceptable formulating agent.

52. (Amended) A composition of Claim 51 wherein said nucleic acid is contained in a viral vector.

53. (Amended) A viral vector comprising a nucleic acid of claim 1.

54. (Amended) A fusion polypeptide comprising an amino acid sequence encoded by the nucleic acid sequence of claim 1 fused to a heterologous amino acid sequence.

55. (Amended) The fusion polypeptide of claim 54 wherein the heterologous amino acid sequence is an IgG constant domain or fragment thereof.

70. (Amended) A nucleic acid according to claim 1 attached to a solid support.

72. (New) The process of claim 8 further comprising isolating the polypeptide from the culture.

APPENDIX C

Biochemical and Biophysical Research Communications 280, 374–379 (2001)
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Isolation, Tissue Distribution, and Chromosomal Localization of a Novel Testis-Specific Human Four-Transmembrane Gene Related to CD20 and FcεRI-β

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CD20 and the β subunit of the high affinity receptor for IgE (FcεRIβ) are related four-transmembrane molecules that are expressed on the surface of hematopoietic cells and play crucial roles in signal transduction. Herein, we report the identification and characterization of a human gene, *TETM4*, that encodes a novel four-transmembrane protein related to CD20 and FcεRIβ. The predicted TETM4 protein is 200 amino acids and contains four putative transmembrane regions, N- and C-terminal cytoplasmic domains, and three inter-transmembrane loop regions. TETM4 shows 31.0 and 23.2% overall identity with CD20 and FcεRIβ respectively, with the highest identity in the transmembrane regions, whereas the N- and C-termini and inter-transmembrane loops are more divergent. Northern blot and RT-PCR analysis suggest that TETM4 mRNA has a highly restricted tissue distribution, being expressed selectively in the testis. Using fluorescence *in situ* hybridization and radiation hybrid analysis, the *TETM4* gene has been localized to chromosome 11q12. The genes for CD20 and FcεRIβ have also been mapped to the same region of chromosome 11 (11q12-13.1), suggesting that these genes have evolved by duplication to form a family of four-transmembrane genes. TETM4 is the first nonhematopoietic member of the CD20/FcεRIβ family, and like its hematopoietic-specific relatives, it may be involved in signal transduction as a component of a multimeric receptor complex. © 2001 Academic Press

Key Words: **four-transmembrane; TM4SF; tetraspanin; CD20; FcεRIβ; testis; gene localization; chromosome 11; signal transduction.**

CD20, the β subunit of the high affinity receptor for IgE, and HTm_4, comprise a family of related proteins that contain four membrane spanning regions. All three proteins are expressed specifically in hematopoietic cells; CD20 on B cells (1), FcεRIβ on mast cells and basophils (2), and HTm_4 on cells of myeloid and lymphoid origin (3). Both CD20 and FcεRIβ have been well characterized as playing important roles in initiating signal transduction events as components of multimeric receptor complexes. CD20 has been shown to have the capacity to regulate B cell proliferation and differentiation as part of a large cell surface complex with MHC-I, MHC-II, CD40, and the tetraspanins CD53, 81 and 82 (1, 4). FcεRIβ is a key component of the tetrameric $\alpha\beta\gamma_2$ FcεRI complex on mast cells and basophils, and plays a crucial role in enhancing cell surface expression of the complex and amplifying signal transduction events mediated upon the interaction of receptor-bound IgE with multivalent allergen (2, 5, 6). The functional role of HTm_4 is unknown, but as for CD20 and FcεRIβ, it is likely to contribute to the signalling of a multimeric receptor complex. In this study, we report the isolation, tissue distribution and chromosomal localization of a human gene that encodes a novel member of the CD20/FcεRIβ/HTm_4 family.

MATERIALS AND METHODS

Isolation of RNA and first strand cDNA synthesis. Total cellular RNA was prepared by homogenising 100 mg of tissue in 1 ml of Trizol reagent (Gibco-BRL, Grand Island, NY), upon which the aqueous fraction was recovered and RNA precipitated using isopropanol. First strand cDNA was produced from 5 μg of total RNA by priming with an oligo dT primer (NotdT, 5′-AACTGGAAGAATTCGCGGCCGCAGGAAT^{18}-3′) using a First Strand cDNA synthesis system (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

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0006-291X/01 $35.00

PCR and nucleotide sequence analysis. PCR was performed on 10 ng of first strand cDNA in the presence of 100 ng of each oligonucleotide primer, 1.25 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 1.5 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD) for 35 amplification cycles. 3'-RACE was performed by PCR as described above with the oligonucleotide primer TET-1 (5'-GTCATCTCCTTTCAAATTATCAC-3', hybridizes to nucleotides 24-46 of the TETM4 cDNA) and the NotdT primer (see above). Nucleotide sequencing was performed by direct sequencing of amplified cDNA fragments using an Applied Biosystems 377 sequencer.

Northern blot analysis. Northern analysis of multiple human tissue blots (Clonetech, Palo Alto, CA) was performed by probing membranes with the full length TETM4 cDNA, labelled by random priming (Megaprime DNA labelling system; Amersham, Buckinghamshire, UK), using Expresshyb solution (Clonetech, Palo Alto, CA) as specified by the manufacturers. Membranes were washed in 1× SSC for 40 min at room temperature followed by 0.1× SSC for 40 min at 60°C and exposed to X-ray film.

Southern blot analysis. 10 μg of genomic DNA was digested with a range of restriction enzymes, separated on a 1% agarose gel, and transferred to a Hybond-N nylon filter (Amersham, Buckinghamshire, UK). The blot was probed with the full length TETM4 cDNA labelled by random priming and hybridized in a 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's solution and 100 μg/ml salmon sperm DNA at 42°C. The membrane was washed in 1× SSC for 40 min at room temperature followed by 0.1× SSC for 40 min at 65°C and exposed to X-ray film.

Fluorescence in situ hybridization. A 1100-bp genomic fragment of the *TETM4* gene, produced by PCR amplification with oligonucleotide primers TET-2 (5'-TTCAACTCAAAGCCCCTTGC-3', hybridizes to nucleotides 155–174 of the TETM4 cDNA) and TET-4 (5'-CCTTGGATATGGTTTTAACAAAG-3', nucleotides 290–268), was nick-translated with biotin-14-dATP and hybridized *in situ* at a final concentration of 15 ng/ml to metaphases from two normal males. The fluorescence *in situ* hybridization (FISH) method was as previously described (7), with the exception that chromosomes were stained before analysis with both propidium iodide (as counterstain) and diaminophenylindole (DAPI) (for chromosome identification). Images of metaphase preparations were captured by a cooled CCD camera using the chromoScan image collection and enhancement system (Applied Imaging Int. Ltd.).

Radiation hybrid analysis. The *TETM4* gene was mapped using the medium resolution Stanford G3 panel of 83 clones. Screening of the panel was performed by PCR amplification of a 1100-bp *TETM4*-specific fragment using oligonucleotide primers TET-2 and TET-4 (see above). Amplifications were performed on 10 ng of each sample DNA under the above conditions.

RESULTS AND DISCUSSION

Identification and Isolation of the TETM4 cDNA

In order to investigate the possible existence of additional novel members of the CD20/FcεRIβ/HTm_4 family, the human dbEST (public expressed sequence tags, GenBank database) was searched with a consensus peptide sequence corresponding to a conserved region of the second transmembrane (TM) region of the three known human family members (GYPFWGAIFFSISG) (3, 8, 9). A number of ESTs were identified, all from testis libraries (GenBank Accession Nos. AI149899, AA416972, AA411806, AA707529, AA470059, AA436088, AA781801, AI002083, AA435988), which contained a region homologous to the conserved search peptide. Sequence analysis of the ESTs suggested that they were fragments of a single gene which was related to, yet distinct from, CD20/FcεRIβ/HTm_4. The EST sequences were assembled into a single contig of 695 bp, and examination of the compiled sequence, suggested an open reading frame that encoded for a putative protein of 200 amino acids. An oligonucleotide primer was designed to the predicted 5' untranslated region of the cDNA (TET-1, 5'-GTCATCTCCTTTCAAATTATCAC-3', hybridizes to nucleotides 24-46 of the TETM4 cDNA) and used in 3' rapid amplification of cDNA ends (RACE)-PCR with the oligo-dT primer NotdT (see Materials and Methods), on first-strand cDNA generated from human testis total RNA. A product of 707 bp was amplified, that upon direct sequencing, was determined to encode the complete coding region predicted from the EST contig, confirming that the cDNA sequence was derived from a single mRNA. The cDNA was also cloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA), and multiple clones were analysed, which revealed an identical sequence to that determined from direct sequencing of the PCR product. The nucleotide and deduced amino acid sequence encoded by the full length cDNA, designated TETM4 (for testis expressed transmembrane-4, see below), is shown in Fig. 1.

The complete TETM4 cDNA is 695 bp long and contains a canonical polyadenylation signal sequence at nucleotides 669–673 (Fig. 1). The cDNA encodes for a deduced protein of 200 amino acids with a predicted molecular weight of 22.2 kDa. Hydropathy analysis indicates the presence of four hydrophobic regions that represent four putative transmembrane domains. Using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html), which predicts membrane spanning regions and their orientation, TETM4 is predicted to have four strong transmembrane helices which are likely to adopt a membrane topology with both the N- and C-termini intracellular. On the basis of this prediction, the TETM4 protein can be divided into the following domains; four transmembrane domains (TM-1, TM-2, TM-3 and TM-4) of 22, 21, 20 and 22 amino acids respectively, N- and C-terminal cytoplasmic domains of 48 and 18 amino acids, respectively, two extracellular loops of 14 and 22 amino acids and a short intracellular loop of 13 amino acids (Fig. 1). Significantly, both CD20 and FcεRIβ have been shown experimentally to have a topology on the cell surface as predicted here for TETM4 (1, 2). However, it should be noted that it remains possible that TETM4 may adopt an alternate topology with both the N- and C-termini extracellular. Furthermore, it also needs to be considered that TETM4 may not be expressed on the cell surface but instead is localized on a subcellular membrane(s).

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-56      GACTAGACTGAAGTACCAACTAAGTCATCTCCTTTCAAATTATCACCGACACCATC

  1  ATGGATTCAAGCACCGCACACAGTCCGGTGTTTCTGGTATTTCCTCCAGAAATCACTGCT
  1  M  D  S  S  T  A  H  S  P  V  F  L  V  F  P  P  E  I  T  A

 61  TCAGAATATGAGTCCACAGAACTTTCAGCCACGACCTTTTCAACTCAAAGCCCCTTGCAA
 21  S  E  Y  E  S  T  E  L  S  A  T  T  F  S  T  Q  S  P  L  Q

121  AAATTATTTGCTAGAAAAATGAAAATCTTAGGGACTATCCAGATCCTGTTTGGAATTATG
 41  K  L  F  A  R  K  M  K  I  L  G  T  I  Q  I  L  F  G  I  M

181  ACCTTTTCTTTTGGAGTTATCTTCCTTTTCACTTTGTTAAAACCATATCCAAGGTTTCCC
 61  T  F  S  F  G  V  I  F  L  F  T  L  L  K  P  Y  P  R  F  P

241  TTTATATTTCTTTCAGGATATCCATTCTGGGGCTCTGTTTTGTTCATTAATTCTGGAGCC
 81  F  I  F  L  S  G  Y  P  F  W  G  S  V  L  F  I  N  S  G  A

301  TTCCTAATTGCAGTGAAAAGAAAAACCACAGAAACTCTGATAATATTGAGCCGAATAATG
101  F  L  I  A  V  K  R  K  T  T  E  T  L  I  I  L  S  R  I  M

361  AATTTTCTTAGTGCCCTGGGAGCAATAGCTGGAATCATTCTCCTCACATTTGGTTTCATC
121  N  F  L  S  A  L  G  A  I  A  G  I  I  L  L  T  F  G  F  I

421  CTAGATCAAAACTACATTTGTGGTTATTCTCACCAAAATAGTCAGTGTAAGGCTGTTACT
141  L  D  Q  N  Y  I  C  G  Y  S  H  Q  N  S  Q  C  K  A  V  T

481  GTCCTGTTCTTGGGAATTTTGATTACATTGATGACTTTCAGCATTATTGAATTATTCATT
161  V  L  F  L  G  I  L  I  T  L  M  T  F  S  I  I  E  L  F  I

541  TCTCTGCCTTTCTCAATTTTGGGGTGCCACTCAGAGGATTGTGATTGTGAACAATGTTGT
181  S  L  P  F  S  I  L  G  C  H  S  E  D  C  D  C  E  Q  C  C

601  TGACTAGCACTGTGAGAATAAAGATGTGTTAAAATAAAAAA
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FIG. 1. Nucleotide and deduced amino acid sequence of human TETM4. The nucleotide sequence is numbered with the first nucleotide of the translational initiation codon as +1. The amino acid sequence is presented below the nucleotide sequence in single letter code, with the four putative transmembrane regions underlined. The predicted initiation codon and stop codon are in bold type and the polyadenylation signal sequence is underlined. GenBank Accession No. AF321127.

Analysis of the TETM4 Amino Acid Sequence

The alignment of the predicted amino acid sequence of TETM4 to that of human CD20 (8), FcεRIβ (9), and HTm_4 (3), indicates an overall identity of 31.0% (55.4% similarity), 23.2% (47.0%) and 26.4% (51.9%), respectively (Fig. 2). The identity of TETM4 to CD20/FcεRIβ/HTm_4 is highest in the transmembrane regions, with the N- and C-termini and intra-transmembrane loop regions showing little homology. TETM4 contains a number of charged/polar residues in its TM regions, including a glutamine residue (Q54) in the first TM domain, an asparagine in each of the second (N97) and third (N121) TM domains, and a glutamic acid (E177) in the forth TM domain. All of these residues, with the exception of N97, are also conserved in CD20/FcεRIβ/HTm_4 (Fig. 2). Interestingly, charged/polar residues are also common in the transmembrane regions of other multi-membrane spanning proteins such as the tetraspanins, which like CD20 and FcεRIβ, associate with other membrane molecules (10). Other interesting structural features of the TETM4 protein include two cysteine residues in its second extracellular loop region (C147 and C156), which are also found in CD20/FcεRIβ/HTm_4, and may be involved in forming an intra- or inter-chain disulphide bond(s). The C-terminal cytoplasmic tail of TETM4 contains a cluster of five cysteine residues in a 12 residue stretch (C189, 194, 196, 199 and 200), which may also be involved in disulphide bond formation.

Identification of a TETM4 Splice Variant

The PCR amplification of the TETM4 cDNA with oligonucleotide primers TET-1 and NotdT (see Materials and Methods) from human testis also led to the isolation of a putative splice variant. Nucleotide sequence analysis indicated that this cDNA is 542 bp long and is identical to the TETM4 cDNA, however, it is missing the coding region for the third trans-

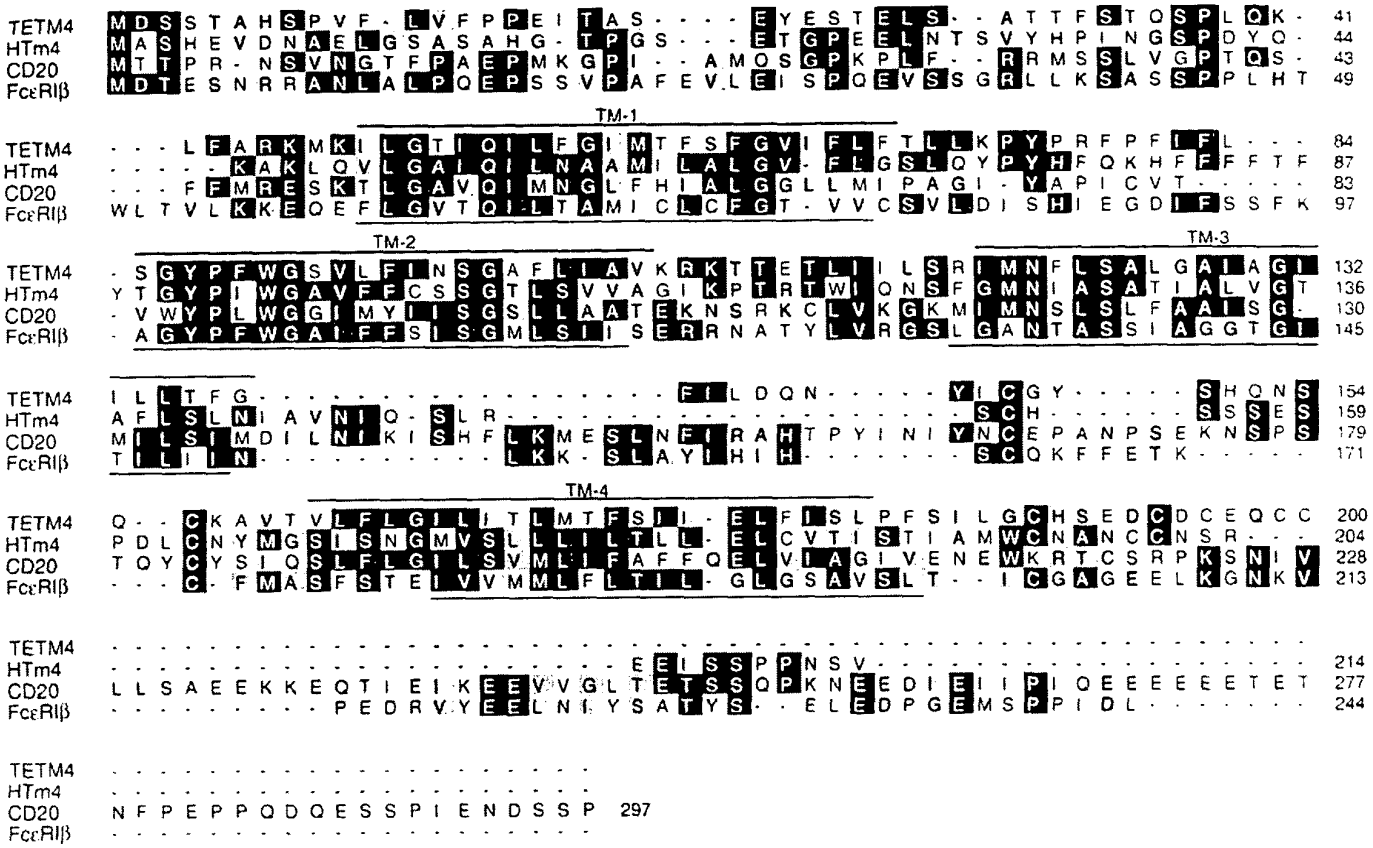

FIG. 2. Amino acid alignment of TETM4 with CD20, FcεRIβ and HTm_4. The amino acid sequences are presented in single letter code. The alignment was performed using CLUSTALW (GCG package) and adjusted manually. Gaps (−) have been introduced to maximise alignment of the sequences. Identical or similar residues between at least two sequences are shaded in black or grey, respectively. Similar residues are defined as: D, E (acidic); A, G, I, L, V (aliphatic); N, Q (side-chain containing amide group; F, W, Y (aromatic); R, H, K (basic); S, T (side-chain containing hydroxyl group). The positions of the four putative transmembrane regions for TETM4 and FcεRIβ are overlined and underlined, respectively. The predicted TM regions for HTm_4 and CD20 are very similar to that shown for FcεRIβ, with the exception that CD20 contains a continuous hydrophobic stretch between TM-1 and TM-2. The GenBank Accession Nos. are: TETM4, AF321127; CD20, AAA35581; FcεRIβ, AAA60269; HTm4, AAA62319.

membrane and second extracellular domains (nucleotides 394 to 547 encoding amino acids L113 to F163). This splice variant is also represented in the EST database by two clones (GenBank Accession Nos. AA411806 and AA781801). The deduced polypeptide encoded by this cDNA would contain only three transmembrane regions, and would therefore be predicted to have a membrane topology with the C-terminal domain extracellular, as opposed to intracellular for the four-transmembrane form. A putative TETM4 protein with this different topology would be likely to have an altered function. The lack of the fourth transmembrane region may influence possible association(s) with other membrane molecules, and the shifting of the C-terminal domain from intracellular to extracellular may change any potential signalling capacity mediated through interactions with intracellular signalling proteins. Clearly, it would be of interest to determine if this variant encodes for a functional protein.

Tissue Distribution of TETM4 mRNA

The tissue distribution of TETM4 mRNA was investigated by Northern blot analysis of a range of human tissues. A strong band centered around 0.7 kb was detected only in testis and not in spleen, thymus, prostate, ovary, small intestine, colon, peripheral blood leukocyte, heart, brain, placenta, liver, lung, skeletal muscle, kidney or pancreas (Fig. 3). Prolonged exposure of the Northern blot failed to reveal any significant signal in any tissue other than testis. Reverse-transcriptase (RT)-PCR analysis of TETM4 mRNA was also performed on first strand cDNA made from mRNA isolated from the above range of human tissues. Amplification with oligonucleotides TET-2 (see Materials and Methods) and TET-3 (5′-CAGTAACAGCCTTAGACTGAC-3′, hybridizes to nucleotides 537–516 of the TETM4 cDNA) produced the expected product of 383 bp only in testis, but not any other tissue (data not shown). These data suggest that TETM4 shows an

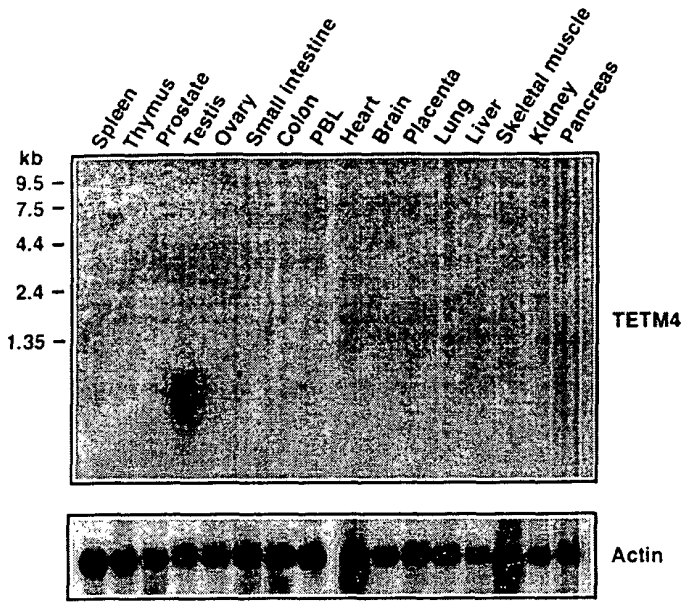

FIG. 3. Northern blot analysis of TETM4 mRNA expression. Multiple tissue Northern blot filters (Clonetech, Palo Alto, CA) were hybridized with ^{32}P-labelled full-length TETM4 cDNA in ExpressHyb solution (Clonetech, Palo Alto, CA) as specified by the manufacturer. The filters were rehybridized with a control ^{32}P β-actin cDNA and show approximately equal amounts of mRNA loaded per lane; heart and skeletal muscle have two β-actin transcripts. The positions of molecular weight makers (in kilobases) are indicated. Exposure times were 12 h for both TETM4 and β-actin.

extremely specific tissue distribution, being found only in the testis. The identification of TETM4 ESTs derived only from human testis libraries, also supports the proposed testis specific expression. Thus, in contrast to CD20/FcεRIβ/HTm$_4$, which are all hematopoietic specific, TETM4 is the first member of this family that is expressed in a non-hematopoietic tissue.

Southern Blot Analysis and Chromosomal Localization of the TETM4 Gene

Southern blot analysis was performed on restricted human genomic DNA using the full length TETM4 cDNA as a probe. A simple banding pattern was produced that is consistent with the *TETM4* gene being a single copy gene (Fig. 4). To determine the chromosome localization of the *TETM4* gene, fluorescence *in situ* hybridization (FISH) was performed on metaphase chromosomes of two normal males using a 1100-bp *TETM4*-specific genomic fragment as a probe. Twenty metaphases from the first male were examined for a fluorescent signal, which was present in all 20 metaphases in the region 11q12-11q13, with 57% of the signal located in the central portion of band 11q12 (Fig. 5). Similar results were obtained from hybridizations of the probe to metaphases from the second normal male. Radiation hybrid mapping of the *TETM4* gene was also performed using the medium resolution Stanford G3 panel of 83 clones. Screening of the panel by PCR amplification of a 1100 bp *TETM4*-specific fragment using oligonucleotide primers TET-2 and TET-4 (see Materials and Methods), indicated that *TETM4* is most closely associated with the Stanford Human Genome Centre marker SHGC-20674, with a LOD score of 13.23 (data not shown). SHGC-20674 is not ordered on a Stanford map; however, Stanford has linked it to marker SHGC-35409 which is ordered on the Stanford Radiation Hybrid Map. The markers most closely associated with *TETM4* are flanked by markers D11S335 and D11S4363. Searches of the Cytogenetic Yac Bank (http://sgiweb.ncbi.nlm.nih.gov/Zjing/yac.html) placed the flanking markers D11S1335 and D11S4363 on Yac WC11.5 which spans the 11q12 cytogenetic band. The genes for CD20, FcεRIβ and HTm$_4$ have also been mapped to the same region of chromosome 11 (11q12-13) (3, 11, 12). These data suggest that the TETM4, CD20, FcεRIβ, and HTm$_4$ genes have evolved by duplication and divergence of the same ancestral gene to form a family of four-transmembrane genes.

The tetraspanins comprise a distinct family of four-transmembrane molecules that are expressed on both hematopoietic and non-hematopoietic cells. In contrast to the CD20/FcεRIβ/HTm$_4$/TETM4 family, the tetraspanins appear to form a far more extensive family and are found in species ranging from schistosomes to humans. At least 20 members have been described in

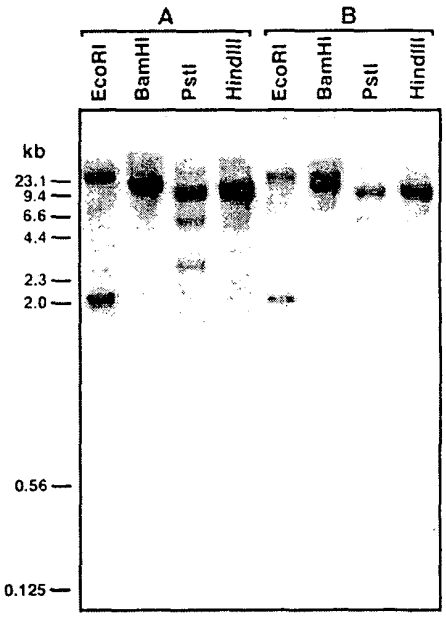

FIG. 4. Southern blot analysis of the *TETM4* gene. 10 μg of human genomic DNA was isolated from two male individuals (A) and (B), restricted with a range of enzymes, and Southern analysis performed by hybridizing with random primed ^{32}P labelled TETM4 cDNA in 50% formamide, 6× SSC, 0.5% SDS and 5× Denhardt's solution. The blot was washed under high stringency conditions and exposed to X-ray film. The positions of molecular weight makers (in kilobases) are indicated.

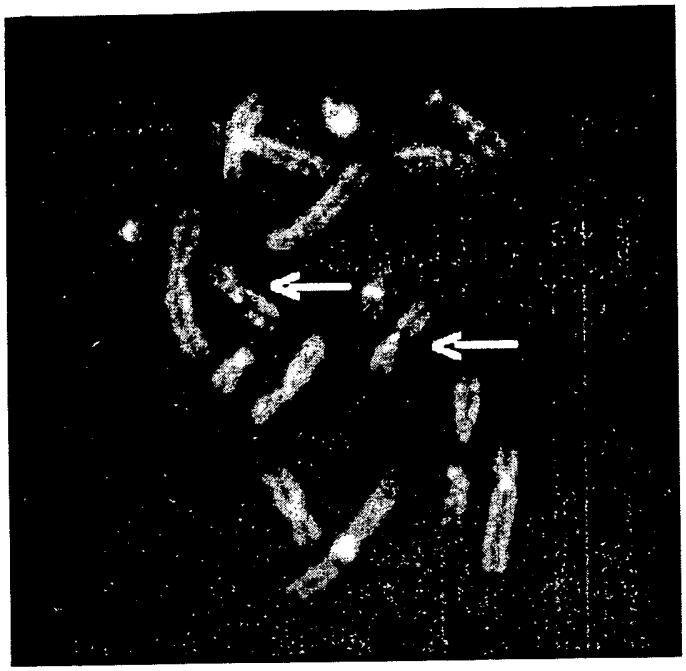

FIG. 5. Chromosomal localization of the *TETM4* gene by FISH. Partial metaphases are displayed showing FISH with a TETM4 probe. Normal male chromosomes have been counterstained with DAPI. Hydridization sites on chromosome 11 are indicated by arrows. FISH signals and the DAPI banding pattern have been merged.

the human, including CD9, CD37, CD53, CD81, and CD82 (10). It is possible that like the tetraspanins, the CD20/FcεRIβ/HTm$_4$/TETM4 family may be much larger. Indeed, we have recently identified a number of additional family members that we are currently characterising (M. Hulett, manuscript in preparation).

The testis-specific expression of TETM4 raises some intriguing questions as to its function. As described above, CD20 and FcεRIβ are expressed specifically on hematopoietic cells where they form components of multimeric cell surface receptor complexes, and play important roles in signal transduction (1, 5, 6). It is therefore tempting to speculate that TETM4 may also associate with receptor complexes on the surface of specific cells in the testis and participate in signalling events. Clearly, to address these possibilities and to delineate the function of TETM4, further fundamental issues need to be addressed such as determining the cellular and subcellular distribution of TETM4 in the testis. These studies are currently in progress.

ACKNOWLEDGMENTS

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APPENDIX D

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Identification of a CD20-, FcεRIβ-, and HTm4-Related Gene Family: Sixteen New MS4A Family Members Expressed in Human and Mouse

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CD20, high-affinity IgE receptor β chain (FcεRIβ), and HTm4 are structurally related cell-surface proteins expressed by hematopoietic cells. In the current study, 16 novel human and mouse genes that encode new members of this nascent protein family were identified. All family members had at least four potential membrane-spanning domains, with N- and C-terminal cytoplasmic domains. This family was therefore named the membrane-spanning 4A gene family, with at least 12 subgroups (*MS4A1* through *MS4A12*) currently representing at least 21 distinct human and mouse proteins. Each family member had unique patterns of expression among hematopoietic cells and nonlymphoid tissues. Four of the 6 human *MS4A* genes identified in this study mapped to chromosome 11q12–q13.1 along with *CD20*, *FcεRIβ*, and *HTm4*. Thus, like CD20 and FcεRIβ, the other MS4A family members are likely to be components of oligomeric cell surface complexes that serve diverse signal transduction functions. © 2001 Academic Press

INTRODUCTION

CD20, high-affinity IgE receptor β chain (FcεRIβ), and HTm4 are three cell surface proteins expressed by hematopoietic cells that represent members of a nascent gene family (Adra *et al.*, 1994; Kinet, 1999; Tedder and Engel, 1994). The deduced amino acid sequence of human and mouse CD20 first demonstrated a cell-surface protein containing four membrane-spanning regions, N- and C-terminal cytoplasmic domains, and an ~50-amino-acid loop that serves as the extracellular domain (Einfeld *et al.*, 1988; Stamenkovic and Seed, 1988; Tedder *et al.*, 1988a,b). Human CD20 shares 20% amino acid sequence identity with FcεRIβ and HTm4 (Adra *et al.*, 1994; Küster *et al.*, 1992). Moreover, these three proteins have a similar overall structure in human, mouse, and rat with significant sequence identity within the first three membrane-spanning domains (Kinet *et al.*, 1988; Ra *et al.*, 1989; Tedder *et al.*, 1988a). In addition, all three genes are located in the same region of human chromosome 11q12–q13.1 (Adra *et al.*, 1994; Hupp *et al.*, 1989; Tedder *et al.*, 1989a) and mouse chromosome 19 (Hupp *et al.*, 1989; Tedder *et al.*, 1988a). These three genes are therefore likely to have evolved from a common precursor.

Despite structural and sequence conservation between CD20, FcεRIβ, and HTm4, transcription of each gene is differentially regulated. CD20 is expressed only by B lymphocytes (Stashenko *et al.*, 1980; Tedder *et al.*, 1988a). FcεRIβ is expressed by mast cells and basophils (Kinet, 1999). HTm4 is expressed by diverse lymphoid- and myeloid-origin hematopoietic cells (Adra *et al.*, 1994). Although the function of HTm4 remains unexplored, CD20 is functionally important for regulating cell cycle progression and signal transduction in B lymphocytes (Tedder and Engel, 1994). Moreover, CD20 forms a homo- or perhaps heterotetrameric complex that regulates Ca^{2+} conductance by either forming or serving as a functional component of a Ca^{2+}-permeable cation channel (Bubien *et al.*, 1993; Kanzaki *et al.*, 1995, 1997a,b). FcεRIβ is part of a tetrameric receptor complex consisting of α, β, and two γ chains (Blank *et al.*, 1989). FcεRI mediates interactions with IgE-bound antigens that lead to cellular responses such as the degranulation of mast cells. Specifically, the FcεRIβ subunit functions as an amplifier of FcεRIγ-mediated activation signals (Dombrowicz *et al.*, 1998; Lin *et al.*, 1996). Because of their unique structure and sequence homologies, CD20, FcεRIβ, and HTm4 are likely to share overlapping functional properties. CD20 and FcεRIβ are also important clinically, as antibodies against CD20 are effective in treating non-Hodgkin's lymphoma (McLaughlin *et al.*, 1998; Onrust *et al.*, 1999; Weiner, 1999). Genetic variations at chromosome 11q12–q13 may also play a role in the

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF237905–237918, AF280401, and AF286866.

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pathogenesis of allergic diseases with *FcεRIβ* representing one candidate gene (Adra *et al.*, 1999; Kinet, 1999).

Since CD20, FcεRIβ, and HTm4 are likely to have evolved by duplication of an ancestral gene, other related proteins that form additional receptor complexes might exist. To address this, we have identified 16 novel human and mouse proteins that span the membrane at least four times and share high levels of amino acid sequence identity with CD20, FcεRIβ, and HTm4. This finding reveals a new gene family that we have designated the MS4A family (membrane-spanning 4-domain family, subfamily A). Currently this family contains at least 12 subgroups (MS4A1 through MS4A12, Table 1) that encode at least 21 heretofore-unidentified human and mouse proteins expressed by hematopoietic cells and diverse cell types in nonhematopoietic tissues.

MATERIALS AND METHODS

Database searches and cDNA isolation. Three hundred thirty-seven nucleotide sequences obtained from the translated GenBank database of expressed sequence tags (ESTs) were assembled into 62 subgroups of contiguous linear segments based on their overlapping sequences and potential for encoding proteins homologous with CD20. Based on these subgroups, EST cDNAs (Fig. 1 and data not shown) were obtained from the American Type Culture Collection (ATCC; Bethesda, MD) and sequenced. Based on the complete sequences of 21 near full-length EST cDNAs, 11 novel genes that unified multiple EST subgroups were defined in human and mouse. Near full-length EST clones representing these genes are shown in Fig. 1. These 11 genes and 5 additional genes were also identified by PCR amplification of transcripts using subgroup-specific primers or primers based on EST sequences. The specific details of how cDNAs representing the 5 genes that were not identified by EST cDNA clones are indicated below. In all cases, ESTs and cDNAs encoding the predicted coding regions of each putative unique gene were sequenced in both directions and at least 2 independent ESTs and/or cDNAs representing near full-length gene products were sequenced. Thereby, there was independent confirmation of accuracy for all of the sequences reported (Fig. 1).

Based on EST subgroup sequences, cDNAs encoding mouse *MS4a4B* and *MS4a4C* were isolated by PCR amplification of C57BL/6 mouse spleen cDNA using both *Taq* and *Pfu* DNA polymerases. Primer sets for *MS4a4B* (sense 5′-CAC GAG GCA CAC AAG CAA AGC-3′, antisense 5′-AAG TGC TTG ACT TAC ATA CTT ACA G-3′) amplified an 879-bp fragment. Primer sets for *MS4a4C* (sense 5′-TGG GTG AGA ACA CAC AAT CAA AAC-3′, antisense 5′-CAC ATA CAC ACA AGA GAA TTA GAC-3′) amplified a 794-bp fragment. EST sequences for *MS4a4D* encoded only the 3′ end of the predicted protein. Since *MS4a4D* sequences were closely related to *MS4a4B* and *MS4a4C* sequences, a sense 5′ primer (5′-CCA TTG TCT GTA CTG TTT CTG CTG-3′) based on consensus *MS4a4B* and *MS4a4C* sequences and a *MS4a4D*-specific antisense primer (5′-GCC AAA TGC ATA CAC ATG TGC AC-3′) were used to amplify a 773-bp fragment from cDNA of C57BL/6 mouse lung.

MS4a6C was initially identified based on one unique EST sequence (AA028258) encoding a mouse protein homologous with the C-terminal end of *MS4a6B*. *MS4a6C* cDNAs were isolated by PCR amplification of C57BL/6 mouse bone marrow cDNA using *Taq* polymerase. A primer based on identical sequences at the 5′ end of the *MS4a6B* and *MS4a6D* cDNAs (sense 5′-CTG GAA GTG ACT GGG TGA CAA GGC-3′) was used in combination with an antisense primer specific for the unique EST sequence (5′-CAA TCG TTC CTC ATA TGC ACA G-3′) to amplify a 787-bp fragment. Sequences from multiple independent PCR-amplified cDNAs were identical. Subsequently, the PCR-generated 5′ end of the near full-length *MS4a6C* cDNA was found to be identical to an orphan EST subgroup sequence that had not been linked with defined 3′ sequences. Thereby, the EST subgroup sequences verified that the PCR-amplified 5′ end of the *MS4a6C* cDNAs was appropriate. In addition, the overall *MS4a6C* sequence was similar to the sequence of *MS4a6B* cDNAs without interruption. Thus, the *MS4a6C* cDNA united sequences identical to those found in two nonoverlapping CD20-homologous EST subgroups.

cDNAs encoding a 473-bp fragment of mouse *MS4a3* were amplified from cDNA of C57BL/6 bone marrow as described above. Primer sets (sense 5′-AGA CTC TGG TGG TCA TTA CTG TCT C-3′, antisense 5′-GAA TGC CAA ATG CAC AGA AAG G-3′) were obtained based on a single thymic cDNA EST sequence (GenBank AA940479) when the corresponding cDNA was not available.

All PCR-amplified cDNAs were subcloned and sequenced entirely in both directions. Complete sequencing of at least two distinct PCR-generated cDNAs from both *Taq* and *Pfu* enzymes was performed in most cases. Differences between cDNA sequences were noted only when multiple cDNA clones generated by both *Taq* and *Pfu* polymerases revealed identical differences. In some cases, cDNAs or EST sequences contained potential intron/exon splice sites that delimited structural domains and aligned with the known intron/exon splice sites of *CD20* (Tedder *et al.*, 1989b). In these cases, potential introns were flanked by consensus splice donor and/or splice acceptor sequences (Aebi and Weissmann, 1987) or were likely to represent splice variants from which exons were deleted.

RNA isolation and reverse transcription-PCR. Reverse transcription-PCR amplification was as described previously (Zhou and Tedder, 1995) with minor modifications. Total RNA was extracted from $1–2 \times 10^7$ cells or frozen tissue using an RNeasy Mini Kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's instructions. RNA concentrations were determined by UV absorbance. Ten micrograms of total RNA was reverse transcribed and PCR amplified with primers (500 nM) for 40 cycles (94°C for 1 min, 55°C for 1.5 min, 72°C for 1.5 min, followed by extension at 72°C for 5 min). Following amplification, the PCR products were separated on 1% agarose–ethidium bromide gels and photographed. G3PDH, a housekeeping gene, was also amplified to control for sample-to-sample variation. RNA amplified without reverse transcription was used as a negative control and was negative in all cases (data not shown).

RESULTS

Identification of CD20 gene family members. To identify new CD20 gene family members, the human and mouse CD20 amino acid sequences (Tedder *et al.*, 1988a,b) were used to search the translated GenBank databases, including expressed sequence tags, using the BLAST program (Altschul *et al.*, 1997). Among 337 homologous sequences identified, at least 17 novel genes expressed by mouse, human, and pig had predicted amino acid sequences homologous to CD20. Complete coding regions were predicted using overlapping nucleotide sequences obtained from sequenced ESTs and cDNAs that corresponded to unique, near full-length transcripts in humans and mice (Fig. 1). All nucleotide sequences were verified by sequencing multiple near full-length cDNAs isolated in our laboratory and 40 cDNAs obtained from the ATCC. In addition, a pig cDNA and its human counterpart homologous to CD20 were identified as GenBank submissions AJ236932.1 and AK000224, respectively. In total, unique cDNA clones that encoded at least 16 distinct full-length CD20-like proteins were identified (Fig. 1).

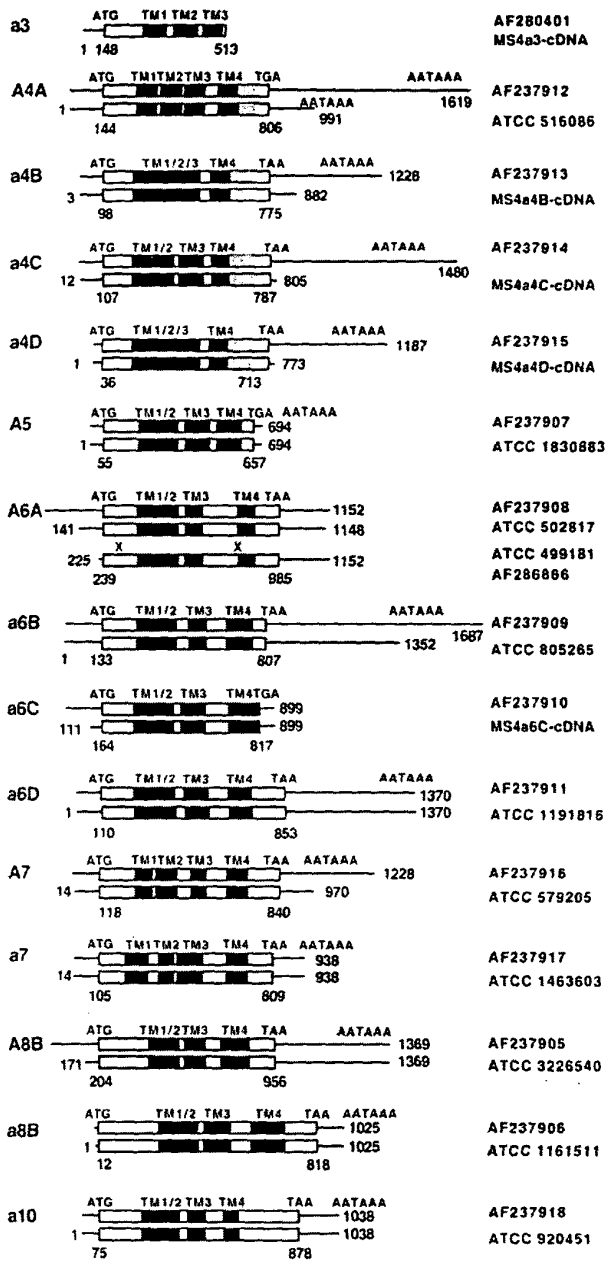

FIG. 1. cDNAs encoded by 15 new human or mouse MS4A gene products. Consensus sequences from cDNAs and overlapping ESTs are indicated by their GenBank accession numbers. Representative full-length cDNAs for each gene product are shown, except for *MS4a3*, which was not full length. 5′ and 3′ untranslated sequences are shown as horizontal lines with relative nucleotide lengths shown. Coding regions are shown as boxes with translation initiation and termination codons and their relative nucleotide locations are shown. Poly(A) attachment signal sequences (AATAAA) are indicated when known. Deduced hydrophobic regions are shown as filled boxes with the predicted membrane-spanning domains shown as TM1–TM4. Additional hydrophobic regions in MS4A4 proteins are shown as shaded boxes. Sites of potential nucleotide polymorphisms in *MS4A6A* are indicated by two X's.

In collaboration with the Human Gene Nomenclature Committee (www.gene.ucl.ac.uk/nomenclature/), this gene family was designated the MS4A family (membrane-spanning 4-domain family, subfamily A). The MS4 designation is to accommodate the future identification of genes encoding proteins with a similar structure, yet with unresolved functions. Subfamily A will designate the *CD20* family. Using this nomenclature, the *CD20* gene was designated *MS4A1*, *FcεRIβ* *MS4A2*, and HTm4 *MS4A3*. Among the 16 novel genes identified, 6 human genes were named *MS4A4A*, *MS4A5*, *MS4A6A*, *MS4A7*, *MS4A8B*, and *MS4A12*. The remaining genes were of mouse or pig origin and were therefore labeled *MS4a3–MS4a12* based on the nomenclature of homologous genes corresponding to human counterparts (Table 1). Distinct mouse gene products that encoded proteins with highly homologous sequences were designated *MS4a4B*, *MS4a4C*, and *MS4a4D* and *MS4a6B*, *MS4a6C*, and *MS4a6D* to signify close homologies.

Structures of MS4A family members. Complete coding region sequences were verified for each deduced protein, except for the *MS4a3* protein, which was not full length (Fig. 1). Proposed ATG translation initiation codons were based on the translation initiation consensus sequence, ANNATG (Kozak, 1986), and the existence of in-frame upstream translation stop codons in most cases. Poly(A) attachment signal sequences were identified in the 3′ untranslated regions of each gene product except *MS4A6A* and *MS4a6C*. Two poly(A) signal sequences were found in *MS4a4D*, *MS4A5*, and *MS4a10* transcripts, while four were observed in *MS4A4A* transcripts. The *MS4A* genes encoded proteins of 22–29 kDa (Fig. 2, Table 1). Whether the first or second ATG codon in mouse *MS4a8B* was used for translation initiation was unknown although the second ATG was identical with the start codon of human *MS4A8B* (Fig. 2). There were no amino-terminal signal sequences, although all MS4A proteins contained hydrophobic regions of sufficient length to pass through the membrane at least four times (Figs. 1 and

TABLE 1

MS4A Family Members

Human		Mouse		
Name	kDa	Name	kDa	Human/mouse homology
		MS4a3		63% (partial)
MS4A4A	23			
		Ms4a4B	24	41%
		Ms4a4C	24	44%
		Ms4a4D	24	40%
MS4A5	22			
MS4A6A	27			
		Ms4a6B	27	52%
		Ms4a6C	24	51%
		Ms4a6D	26	53%
MS4A7	26	MS4a7	26	53%
MS4A8B	26	MS4a8B	29	63%
		MS4a10	29	
MS4A12	26	MS4a12 (pig)	26	60%

Note. Predicted molecular weights for the new MS4A family members and the percentage amino acid sequence identity between deduced MS4A and MS4a proteins.

FIG. 2. Deduced amino acid sequences for CD20 (human A1, mouse a1), FcεRIβ (A2 and a2), HTm4 (A3 and a3), and 16 new MS4A (human) and MS4a (mouse and pig) proteins. Gaps were introduced to optimize alignments. Numbers represent predicted residue positions. The predicted membrane-spanning regions (TM) are indicated. Predicted intron/exon splice junctions are indicated by vertical bars where information was available. Amino acids common to 10 or more proteins are shaded. *Partial sequence for the MS4a3 protein. CD20, FcεRIβ, and HTm4 sequences and known intron/exon borders are as published (Adra *et al.*, 1994; Küster *et al.*, 1992; Ra *et al.*, 1989; Tedder *et al.*, 1988a,b, 1989b). MS4A12 represents a human colon mucosa cDNA sequence (GenBank AK000224) and MS4a12 represents a homologous cDNA sequence from pig (GenBank AJ236932).

2). Notable was a marked clustering of charged residues at both ends of the postulated transmembrane domains, some of which were highly conserved. In some cases, the first and second putative transmembrane domains of MS4A proteins were a continuous stretch of hydrophobic amino acids without an obvious intertransmembrane hydrophilic bridge. In contrast, *MS4A4A* and *MS4A7* had 6 to 7 hydrophilic amino acids inserted between the first and the second hydrophobic domains. In human *MS4A4A* and mouse *MS4a4B*, *MS4a4C*, and *MS4a4D*, an extensive hydrophobic region followed the fourth proposed membrane-

spanning domain. Whether this region allows the protein to traverse the membrane a fifth time is unknown. Nonetheless, the overall structure of MS4A family members was well conserved.

Comparisons between CD20 and the predicted amino acid sequences for human *MS4A4A, MS4A5, MS4A6A, MS4A7, MS4A8B,* and *MS4A12* revealed 23–29% amino acid sequence identity (Fig. 2). The highest degree of identity was found in the first three transmembrane domains with multiple regions of conserved amino acids. In particular, the amino acid sequences LGAXQI and LSLG were common within the first transmembrane domain, GYPFWG and FIISGSLS were common in the second domain, and SLX_2NX_2 SX_3AX_2G was found in the third transmembrane domain. The first and second transmembrane domains of *MS4A8B* were 46% identical in amino acid sequence with human CD20, 41% identical with FcεRIβ, and 39% identical with HTm4. The *MS4A4A, MS4A5, MS4A6A,* and *MS4A7* proteins were most homologous in their first and second transmembrane domains with the human FcεRIβ chain, with 37–46% amino acid sequence identity. There was large variation between MS4A proteins in the N- and C-terminal cytoplasmic domains. However, Pro residues were significantly overrepresented within the N- and C-terminal cytoplasmic domains of most MS4A family members. There was some sequence identity in the first potential extracellular loop that was ~13 amino acids in length for each protein. In contrast, the second predicted extracellular loop ranged from 10 to 46 amino acids in length with diverse sequences.

Potential polymorphisms were identified in the *MS4A6A* gene. Two nucleotide substitutions were found in cDNA clone ATCC 499181 and in 13 of 38 EST sequences analyzed (Fig. 1). The first substitution was at nucleotide 373 and exchanged a C for a T, which did not alter the amino acid sequence. The second substitution resulted in a Ser in place of Thr at amino acid 185. In addition, a third substitution was found in 4 of the 38 EST sequences analyzed, in which a Ser was substituted in place of an Ala at amino acid position 183. This substitution was paired with a Ser-to-Thr substitution at amino acid position 185 in half of the clones analyzed. These differences most likely represent common sequence polymorphisms since they were observed in multiple independent cDNA clones. Although less likely, these differences could represent transcripts from distinct genes that are almost identical in coding sequence. Other potential polymorphisms were observed in other MS4A family members based on consistent nucleotide variations found in large numbers of overlapping EST sequences. However, we were unable to independently verify the existence of these sequence polymorphisms through the isolation and sequencing of independent cDNAs bearing each potential polymorphism.

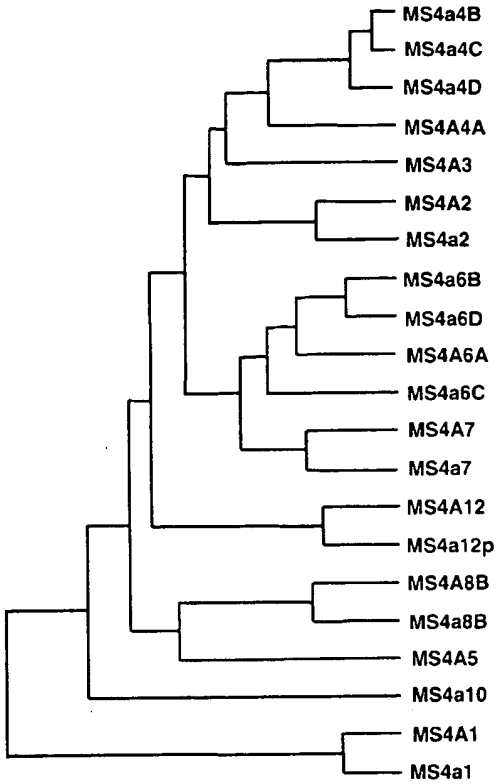

FIG. 3. UPGMA (unweighted pair group method using arithmetic averages) tree of deduced MS4A and MS4a protein sequences. Horizontal tree branch length is a measure of sequence relatedness. For example, *MS4a4B* and *MS4a4C* are the most similar in sequence, while *CD20* (*MS4A1*) sequences were the most divergent from other family members. The *MS4a12p* sequence was from pig, while all other MS4a sequences were from mouse. The UPGMA tree was generated using Geneworks version 2.0 (IntelliGenetics, Inc., Mountain View, CA).

Mouse MS4A proteins. Ten mouse MS4A proteins that shared 40–63% amino acid sequence identity with their potential human counterparts were identified (Fig. 2, Table 1). For comparison, the mouse and human CD20 proteins are 74% identical in amino acid sequence (Tedder *et al.*, 1988a). A single partial cDNA that encoded the mouse homologue for HTm4 was identified (*MS4a3;* Fig. 2). The predicted amino terminus of the proposed *MS4a3* protein was 23 amino acids shorter than in the human protein, although their overlapping regions were 63% identical in amino acid sequence. In all cases, the transmembrane domains of the human and mouse MS4A proteins were the most well conserved regions. For example, the human *MS4A8B* protein was 78% identical in sequence to *MS4a8B* in the first three transmembrane domains and 68% identical in domain 4. The mouse *MS4a10* protein shared little sequence homology with any of the human MS4A proteins (Fig. 3). Therefore, additional *MS4A* genes are likely to be identified in humans and mice, including the mouse *MS4A5* and human *MS4a10* counterparts.

Expression of MS4A family members. Since CD20, FcεRIβ, and HTm4 expressions are restricted to hematopoietic tissues, MS4A gene transcription was assessed by PCR amplification of cDNA from 11 human

TABLE 2

MS4A mRNA Expression by Human Lymphoblastoid Cell Lines

	MS4A family member[a]									
Cell line	1	2	3	4A	5	6A	7	8B	12	G3PDH
Pre-B										
NALM-6	−	−	−	+++	−	−	−	−	−	+++
B cell										
BJAB	+++	−	−	+++	−	−	+++	+	−	+++
DAUDI	+++	−	−	+	−	−	+++	+	−	+++
SB	+++	−	−	++	−	+++	+++	+	−	+++
T cell										
HSB-2	−	−	−	+	−	−	−	−	−	+++
HUT-78	−	−	−	+	−	−	+	−	−	+++
JURKAT	−	−	−	+	−	−	−	−	−	+++
MOLT15	−	−	−	+	−	−	++	−	−	+++
Myelomonocyte										
HL60	−	−	+++	++	−	+++	+++	−	−	+++
U937	−	−	+++	+++	+	+	+++	−	−	+++
Erythroleukemia										
K562	−	+	+++	+++	−	+	−	−	−	+++

[a] Gene transcription was assessed by PCR amplification of cDNA generated from mRNA isolated from each cell type. Values represent the level of PCR product generated relative to the G3PDH control in three separate PCR: −, no specific PCR product detected: +, low levels of the appropriate band were detectable; ++ to +++, appropriate bands of increasing intensity were readily visualized in all samples examined. Identical results were obtained using two different primer pairs for cDNA amplification

hematopoietic cell lines. Like *CD20, MS4A8B* was expressed only by B cell lines (Table 2). *MS4A5* was expressed only by a promonocytic cell line. *MS4A6A* transcripts were expressed by B, myelomonocytic, and erythroleukemia cell lines. *MS4A4A* mRNA was expressed by all cell lines examined, although the relative mRNA levels varied significantly. *MS4A7* was expressed in most, but not all, of the cell lines tested. *MS4A12* transcripts were not detected in these cell lines. Thus, most MS4A family members are likely to be expressed in hematopoietic tissues.

ESTs encoding MS4A transcripts were isolated from a variety of different cDNA libraries. *MS4A4A* ESTs were from aorta, brain, breast, heart, kidney, lung, ovary, pancreas, placenta, prostate, stomach, testis, and uterine tissues. *MS4A5* ESTs were isolated only from testis. *MS4A6A* ESTs were from aorta, brain, central nervous system, colon, gall bladder, heart, kidney, lung, muscle, ovary, pancreas, placenta, prostate, skin, stomach, tonsil, uterus, and embryonic tissues. *MS4A7* ESTs were from lung, kidney, lymphocytes, mammary gland, placenta, spleen, testis, thymus, and uterine tissues. *MS4A8B* ESTs were from brain, lung, uterus, and embryonic tissues. A single *MS4A12* EST was isolated from colon. This demonstrates differential *MS4A* gene transcription among lymphoid and nonlymphoid tissues.

MS4a gene expression by mouse tissues was assessed by Northern analysis and PCR amplification of cDNAs (Table 3). In most cases assessed, Northern analysis failed to detect specific *MS4a* transcripts in tissues that revealed transcript production by PCR amplification (data not shown). These results suggest that MS4a transcripts are produced only by subpopulations of cells within each tissue such that transcript levels were often below the level of detection by Northern analysis. Nonetheless, *MS4a4B, MS4a4C,* and *MS4a6B* transcripts were found at high levels in thymus, spleen, and peripheral lymph nodes, with less abundant levels in nonlymphoid tissues. *MS4a6C* was expressed only by thymus, spleen, PLN, and bone marrow. *MS4a4C, MS4a6D,* and *MS4a7* were expressed in all tissues examined. *MS4a8B* transcripts were expressed by spleen, peripheral lymph nodes, colon, liver, heart, lung, and bone marrow. *MS4a10* transcripts were found in thymus, kidney, colon, brain, and testis. In addition, *CD20* (*MS4a1*), *FcεRIβ* (*MS4a2*), and *MS4a3* expressions were primarily restricted to hematopoietic tissues. *MS4a3, MS4a4B, MS4a4C, MS4a6B, MS4a6C, MS4a6D, MS4a7, MS4a8B,* and *MS4a10* were also expressed by various hematopoietic and lymphoblastoid cell lines (data not shown). Therefore, most MS4a family members were expressed by hematopoietic cells.

MS4A gene chromosome localization. Chromosome locations for the human *MS4A4A, MS4A6A, MS4A7,* and *MS4A8B* genes were identified in two distinct homology searches. Regions of human *MS4A4A* (bp 1286–1588), *MS4A6A* (bp 682–1106), *MS4A7* (bp 502–941), *MS4A7* (bp 1015–1177), and *MS4A8B* (bp 1007–1350) (Fig. 1) were 98, 98, 97, 99, and 97% identical with human STS genomic sequence tag sites WI-11578, SHGC-36634, WI-12101, WIAF-3856, and WI-14145, respectively (http://www.ncbi.nlm.nih.gov/blast). These genomic sequence tag sites are located on human chromosome 11 at Genomic Database locus D11S1357–D11S913, which maps to 11q12–q13

TABLE 3

MS4a Gene Expression by Mouse Tissues[a]

	mRNA expression										
MS4a	Thymus	Spleen	PLN	BM	Liver	Kidney	Heart	Colon	Lung	Brain	Testes
1	+	+++	+++	+	−	−	−	−	+	−	−
2	+	+	+	+++	−	+	−	−	+	−	−
3	+	+	+	+++	−	−	−	−	+	+	−
4B	+++	+++	+++	++	+	+	+	+	+	−	−
4C	+++	+++	+++	+++	+	+	+	+	+	+	+
4D	+	+	++	−	+	+	++	++	++	−	+
6B	+++	+++	+++	++	+	−	+	+	+	−	++
6C	+	+	+	++	−	−	−	−	−	−	−
6D	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++
7	++	++	++	++	+	+	+	++	+	−	−
8B	−	+	+	+	+	−	+	++	+	−	−
10	+	−	−	−	−	+	−	+	−	+	++
G3PDH	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

[a] Gene transcription was assessed by PCR amplification of cDNA generated from mRNA isolated from tissue samples. Values represent the level of PCR product generated relative to the G3PDH control as described for Table 2. PLN, peripheral lymph node; BM, bone marrow.

(http://www.ncbi.nlm.nih.gov/genemap). These mapping results were confirmed using the UniGene collection at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Genemap98/) for expressed sequence tags identical to human *MS4A4A, MS4A6A, MS4A7,* and *MS4A8B* sequences. Thus, at least seven of the nine currently identified human MS4A genes are clustered.

DISCUSSION

Sixteen novel genes that encoded proteins sharing structural and sequence homologies with *CD20* (*MS4A1*), *FcεRIβ* (*MS4A2*), and *HTm4* (*MS4A3*) were identified (Fig. 3). Together, these proteins demonstrate the existence of a new gene family that we have termed MS4A. All the MS4A proteins contain four potential membrane-spanning domains with both N- and C-terminal cytoplasmic domains. Although the MS4A proteins are structurally similar to other membrane-spanning proteins with four transmembrane domains, their sequences are distinct from any previously known gene family. All MS4A proteins are similar in size, with *MS4A5* (200 amino acids) being the smallest and human CD20 the largest (Fig. 2). Moreover, 7 of the 9 currently identified human MS4A genes (*MS4A1, MS4A2, MS4A3, MS4A4A, MS4A6A, MS4A7,* and *MS4A8B*) were located at chromosome 11q12–q13.1. In addition, *MS4A1, MS4A2, MS4a4B, MS4a4C, MS4a6B,* and *MS4a6C* had predicted intron/exon boundaries at similar positions within their deduced proteins (Fig. 2). These features demonstrate that these genes evolved from a common precursor and share a close evolutionary history (Fig. 3).

Among the human and mouse MS4A proteins, the most significant homologies between family members were found in the first three membrane-spanning domains (Fig. 2). Common amino acid motifs were readily visualized, such as KXLGAIQI, GYPXWG, and SGX-LSI, in the first and second transmembrane regions. In the third and fourth membrane-spanning domains, conserved amino acids were appropriately spaced such that one face of the potential α-helical protein is highly conserved in sequence (Fig. 2). The numbers of highly conserved residues in the membrane-spanning domains and the high degree of sequence conservation at the amino acid level between human and mouse family members suggest that the transmembrane regions play a critical role in MS4A protein function.

Outside of the transmembrane regions, there is considerable diversity in size and sequence of the MS4A proteins (Fig. 3). The predicted second extracellular loops that form the bulk of the extracellular domains of these proteins showed little or no sequence homology between family members. This suggests that this domain can tolerate many changes without affecting any possible functional role or that this domain has undergone significant divergence to serve specific functions. The N- and C-terminal cytoplasmic domains were also divergent between family members except that they were Pro rich in most cases. The basis for this unusual feature is unknown, but suggests that these domains may display unique structural and/or functional characteristics. Surprisingly, CD20 was the most divergent family member (Fig. 3). In contrast, the three members of the MS4a4 and three members of the MS4a6 subfamilies were highly conserved (70–84% of the amino acids were identical within the mouse MS4a4 and MS4a6 subfamily, with MS4a4B and MS4a4C the most similar in sequence). Since only single human counterparts for mouse MS4a4 and MS4a6 subfamilies were found, additional MS4A genes are likely to exist. Therefore, this family of transmembrane proteins may be larger than is currently appreciated.

Two common sequence polymorphisms were identified in the MS4A6A gene (Fig. 1), with a third likely.

Polymorphisms in other MS4A family members are also likely to exist since there were consistent substitutions observed among multiple EST sequences. However, we were unable to isolate independent cDNAs in these cases corresponding to each potential polymorphism. Nonetheless, atopic allergies have been linked to human chromosome 11q12–q13 (reviewed in Kinet, 1999), on which at least seven of the nine currently identified human MS4A genes reside. Specifically, several coding sequence polymorphisms in the *MS4A2* gene have been associated with allergic phenotypes. However, the involvement of other MS4A members in this process cannot be ruled out since MS4A polymorphisms alone may not account for all features of asthma and allergies (Adra *et al.*, 1999; Furumoto *et al.*, 2000; Mohan *et al.*, 1999). The further characterization of additional MS4A family members may provide information for understanding immunologic abnormalities that may lead to the development of atopy and other allergic responses. This is particularly relevant since many of the novel MS4A family members identified in the current study were preferentially expressed by cells of the immune system.

CD20, FcεRIβ, and HTm4 expressions are restricted to discrete hematopoietic cell subpopulations and tissues. However, expression of the new MS4A family members was diverse among human and mouse tissues, although most family members were expressed by hematopoietic tissues and cell lines (Tables 2 and 3). For example, CD20 is B cell restricted, while *MS4A4A* is broadly expressed by most hematopoietic lineages and tissues (Table 2). Among hematopoietic cells, the MS4A genes were differentially regulated during lineage commitment and/or maturation. For example, *MS4a6D* was broadly expressed by lymphoid and nonlymphoid tissues, but numerous hematopoietic cell lines did not express these transcripts. Even among closely related subfamily members, such as *MS4a6B*, *MS4a6C*, and *MS4a6D*, there were considerable differences in expression patterns among tissues and cell lines. Therefore, it is likely that the MS4A proteins will have functions in multiple diverse cell types in addition to hematopoietic tissues.

CD20 forms homo- or hetero-oligomeric complexes (Bubien *et al.*, 1993; Tedder and Engel, 1994) and FcεRIβ forms tetrameric complexes with α and γ chains (Blank *et al.*, 1989). Other MS4A family members may be involved in the formation of similar multimolecular complexes. Given this, the tetrameric CD20 complex may be composed of CD20 and other members of the MS4A family since they are all of similar size (Table 1). This is supported by the finding that B lymphocytes and most other cell types express multiple MS4A family members (Tables 2 and 3). Thus, the MS4A family may resemble the GABA-A receptor gene family. GABA-A receptors are ligand-gated ion channels generated by the assembly of five individual subunits that are structurally similar to MS4A family members (Whiting, 1999). GABA-A receptor subunits have four transmembrane domains containing conserved amino acids with a large protein loop between the third and the fourth transmembrane domains. Assembly of structurally similar, but distinct receptor subunits in differing stoichiometries generates GABA-A receptors with differing pharmacological properties (Sieghart *et al.*, 1999). MS4A family members may assemble to generate similar heterologous complexes. Nonetheless, the sequence homologies within the transmembrane domains of MS4A family members suggest that these proteins have functional properties similar to CD20 or FcεRIβ subunits that function either directly as ligand-gated ion channels or as essential components of receptor complexes. Given that anti-CD20 monoclonal antibodies are effective immunotherapeutics for human malignancies, other MS4A family members may also serve as targets for therapeutic intervention.

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APPENDIX E

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Original Article

Chromosome 11q13 and atopic asthma

Adra CN, Mao X-Q, Kawada H, Gao P-S, Korzycka B, Donate JL, Shaldon SR, Coull P, Dubowitz M, Enomoto , Ozawa A, Syed SA, Horiuchi T, Khaeraja R, Khan R, Lin SR, Flinter F, Beales P, Hagihara A, Inoko H, Shirakawa T, Hopkin JM. Chromosome 11q13 and atopic asthma
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Asthma is a complex syndrome in which bronchial inflammation and smooth muscle hyperactivity lead to labile airflow obstruction. The commonest form of asthma is that due to atopy, which is an immune disorder where production of IgE to inhaled antigens leads to bronchial mucosal inflammation. The ultimate origins of asthma are interactive environmental and genetic factors. The genetics is acknowledged to be heterogeneous, and one chromosomal region of interest and controversy has been 11q13. To clarify the nature of the chromosome 11q13 effect in atopy and asthma, we conducted a genetic association study in subjects with marked atopic asthma and matched controls, which incorporated the study of 13 genetic variants over a distance of 10–12 cM and which took account of detailed immune and clinical phenotyping. Association with high IgE levels was limited to the interval flanked by D11S1335 and CD20 in a 0.8-Mb interval and was greatest for variants of FcεRIβ and HTm4; these variants also associated with asthma (recurrent wheeze with labile airflow obstruction and need for regular inhaler treatment). At the more telomeric marker, D11S480, variants associated with asthma, but not with high IgE levels. The data might support the possibility of multiple loci relevant to atopic asthma on chromosome 11q13.

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Key words: association study – asthma – atopy – linkage disequilibrium – polymorphism

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Atopy is a common disorder characterized by increased general IgE responsiveness (1). Atopy is also an important cause of disorder in the skin (eczema), lung (asthma), and the nose (rhinitis), and family studies suggest variable combinations of organ-specific clinical syndromes in individuals within atopic families (1).

A significant portion of atopic asthmatic families in Caucasian populations may be linked to chromosome 11q13 through the maternal line (2). Data

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from Japan (3) and Germany (4), using lod scores, and from the Netherlands (5), using affected sib-pair methods, have confirmed linkage in families with marked atopy irrespective of clinical symptoms. The β subunit of the high affinity IgE receptor (FcεRIβ) gene has been mapped to chromosome 11q13.1 (6, 7) and is a candidate gene for atopy because of its important role in initiating type I allergic reaction by mast cells and basophils. A recent large-scale population-based linkage study by sib-pair methodology affirms linkage of asthma with microsatellite repeats of FcεRIβ, but not with other markers on 11q13, in an Australian population (8).

The FcεRIβ gene is composed of seven exons and six introns, spanning approximately 11 kb (9). Six variants of this gene have been identified, including three coding and three non-coding variants. Leu181Ile/Leu183Val variants of FcεRIβ have been identified in some British (10) and Australian (11) asthmatic families and showed significant association with atopic asthma (12, 13). Another coding variant, Gly237Glu, is also associated with atopy and/or asthma in Australian, British (14) and Japanese (15) populations. Three non-coding variants, including $(CA)_n$ in the fifth intron (16) and two *Rsa*I restriction fragment length polymorphisms (RFLPs) in the second intron (17) and in the 3′ untranslated region of exon 7 (16), also showed strong genetic association with heightened IgE responsiveness, suggesting the candidacy of FcεRIβ as an important cause for atopic asthma (18).

Recently, the gene encoding the four-transmembrane protein, HTm4, has been cloned and mapped to the same chromosome 11q13 region by our group (19). The high structural and topological homology (24–60% at transmembranous portions) to CD20 and FcεRIβ enables us to propose that HTm4, FcεRIβ, and CD20 evolved from the same ancestral gene to form a family of four-transmembrane proteins (19). Since this family is selectively expressed in hematopoietic linages, *HTm4* is also a candidate locus for type I allergic disorder.

Despite the accumulation of data in which FcεRIβ associates with atopy, genetic linkage or association for atopic asthma has been found on 11q13.1 with other markers *FGF3* (20), D11S534 (21), or D11S97 (22) (in relation to total serum IgE level), or D11S527 (21) or *CC16* (renamed from *CC10*) (23) in relation to asthma or bronchial hyper-responsiveness. Others have not found association nor linkage for atopic asthmatic phenotypes with any genes or markers on the 11q13.1 region (24–31). This area of research, therefore, still remains controversial because of genetic heterogeneity or differences in phenotype assignments among researchers.

The aim of this research is, therefore, to clarify the localization of atopy and/or asthma using polymorphic genes or microsatellite markers that span the entire 11q13.1 region (Fig. 1) in a freshly collected random case–control population from Oxfordshire, where the original linkage to 11q13 was found.

Materials and methods

Subjects selection

One hundred and fifty pregnant women were drawn as controls from the Obstetrics Clinic in Oxford as a general population control (32). One hundred and twenty-five patients with atopic

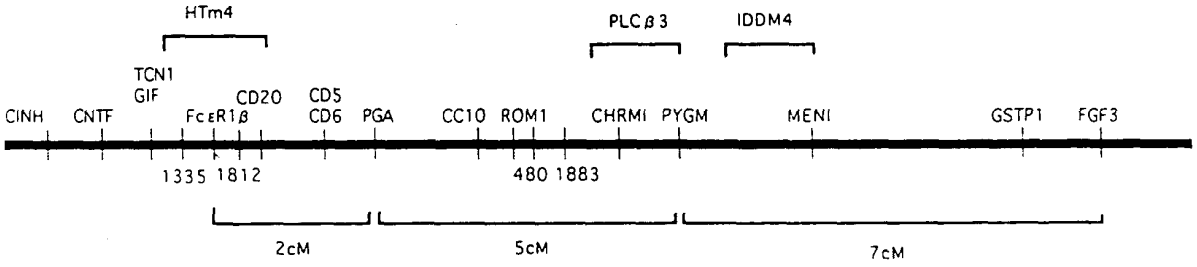

Fig. 1. Schematic presentation of genetic distance on the whole region of 11q13. The left and right ends are centromeric and telomeric, respectively. Estimated distances are based on references (34, 38, 52). Symbols on the line are genes and under the line are microsatellite markers. The exact localization of Kappa, UCP2, D11S527, and D11S534 remains undermined, though EST of Kappa is localized telomeric to PYGM; C1NH; complement component 1 inhibitor, CC10 (CC16); clara cell 10, CHRM1; cholinergic receptor, muscarinic 1, CNTF; ciliary neurotrophic factor, FGF3; fibroblast growth factor 3, GIF; gastro intrinsic factor, GSTP1; glutathione S-transferase pi, HTm4; human transmembranous protein 4, IDDM; insulin-dependent diabetes mellitus, MEN1; multiple endocrine neoplasia 1, PGA; pepsinogen A, PLCβ3; phospholipase Cβ3, PYGM; phosphorylase, glycogen; muscle, ROM1; retinal outer segment membrane protein 1, TCN1; transcobalamin 1.

Table 1. Association of variants in ten genes and three microsatellite markers on 11q13 with atopy or asthma phenotype

Gene	Distance from GIF (cM)[a]	Polymorphism	Marked asthma	Marked atopy
			Odds[b] (95%CI)	Odds[b] (95%CI)
GIF		Intron 4, *Dra*III RFLP	1.28	1.01
D11S1335	0.2	$(CA)_n$, 173 bp[c]	1.48	1.33
HTm4	0.4	Intron 2, *Taq*I RFLP	3.13 (1.41–8.88)**	3.31 (1.14–13.99)*
FcεRIβ[d]	0.4	Intron 2, *Rsa*I RFLP	3.37 (1.88–7.97)**	4.77 (2.45–10.77)**
CD20	1.0	Intron 4, *Sca*I RFLP	1.90	1.13
CC16/CC10	3.5	5′ promotor, *Sau*96I RFLP	1.85	1.45
ROM1	4.5	Intron 1, *Mse*I RFLP	1.68	1.65
CHRM1	5.0?	A1494G, *Pvu*II RFLP	1.88	1.10
D11S480	5.5	$(CA)_n$, 201 bp[c]	2.77 (1.18–7.04)*	1.89
D11S1883	6.0	$(CA)_n$, 262 bp[c]	1.32	1.23
Kappa	7.0	Intron 192–4, *Apa*I RFLP	1.56	1.39
GSTP1	10.0	Ile105Val, *BsmA*I RFLP	1.10	1.11
UCP2	12.0	A55V, *Bbv*I RFLP	1.97	1.96

[a] Estimated distance on the basis of references.
[b] Odds against control subjects.
[c] Allele with highest odds.
[d] *Rsa*I RFLP in the second intron.
* $p<0.05$.
** $p<0.01$.

asthma were collected from the Osler Chest Unit in Oxford; all were Caucasians (32).

All the asthmatic subjects had specialist physician-diagnosed asthma with 1) recurrent breathlessness and chest tightness requiring on-going treatment, 2) physician documented wheeze, 3) documented labile airflow obstruction with variability in serial peak expiratory flow rates greater than 30%. They showed a positive skin prick test of greater than 5 mm against any common antigens or a positive IgE (>0.7 IU/ml) in serum. Marked asthma was designated as chronic rather than episodic asthma and physicians' use of multidrug therapy with steroid inhalers. The disease of atopy was considered by IgE serology (see below). There were no heavy smokers (>20 cigarettes per day) in these subjects.

Serological analysis

Specific IgE against house dust mite (HDM) and grass pollen mix (GX) was detected by the CAP system (Pharmacia, Uppsala, Sweden). The criteria for a positive titer of allergen-specific IgE were as used previously (2, 6). A high total IgE by CAP system was taken to be greater than published normal values for children or greater than 120 kU/l (mean + 1 SD) in adults (2, 6). Atopy, defined as IgE responsiveness, was diagnosed as the presence of a high concentration of total serum IgE, a positive specific IgE titer against one or more aero-allergens, or a combination of these two features.

DNA analysis

DNA samples were extracted using a commercial kit (IsoQuick, Microprobe Corporation, Garden Grove, USA). The polymorphic microsatellite repeats for D11S480, D11S1335, and D11S1833 were amplified from genomic DNA with rhodamine-labeled primers by polymerase chain reaction (PCR), and images were obtained by scanning in 6% polyacrylamide gel with a fluorescent image analyzer, ABI prism 310 (Perkin Elmer, USA). RFLPs for the ten loci, UCP2, ROM1, CHRM1, GSTP1, Kappa, CC10/CC16, GIF, CD20, HTm4, and FcεRIβ were studied. Primers and PCR conditions were described elsewhere for GIF (17), CD20 (17), CC16 (23, 32), CHRM1 (33), Kappa (34), ROM1 (35), GSTP1 (36), UCP2 (37), and FcεRIβ at Gly237Glu (15) and in intron 2 (17) and 3′ tail (16). Primers for new polymorphisms were 5′CCGATTGGGGTGCTGTGT and 5′ATTCAGCTCTGGGGCACCA for HTm4, and 5′TGGGGACAATTCCAGAAGATT and 5′TCCTGTGGGAGAGCAAGATT for FcεRIβ in the 5′ promoter region. PCR in a mixture including 1.5 mmol/l of magnesium chloride in 100 ml was performed in a Perkin Elmer Cetus thermal cycler using a preliminary cycle (94°C for 5 min) and then 34 cycles for HTm4 (94°C for 30 s, 58°C for 60 s, and 72°C for 90 s) or for FcεRIβ in the 5′ promoter (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s). Amplification products were digested with *Taq*I for HTm4, *Bbv*I for UCP2, *Sca*I for CD20, *Dra*III for GIF, *Mse*I for ROM1, *BsmA*I

for GSTP1, *Apa*I for Kappa, and *Sau*96I for CC16 (Table 1). For FcεRIβ, *Mse*I for the 5′ promoter region, *Rsa*I for intron 2 and exon 7 untranslated tail (Table 1), and *Xmn*I for Gly237Glu were studied. The polymorphic microsatellite repeats for D11S1335, D11S1883, and D11S480 were amplified from genomic DNA with fluorescent forward-primers in a PCR, and images were obtained by scanning in 6% polyacrylamide gel with an automated sequencer (ABI 310). Sequencing was conducted with a big-dye system (ABI, UK) using downstream primers, and images were visualized in the commercialized POP-6 gel using the automated sequencer (ABI prism 310 Genetic Analyzer).

Statistics

Contingency table analysis, odds ratios (ORs), 95% confidence intervals (CI), and significance values were estimated by computerized exact methods (SPSS program). OR was calculated between high and low risk genotypes in genes and between high and low risk alleles in markers.

Results

The genotypes for newly identified RFLPs for HTm4 (*Taq*I) and for FcεRIβ (*Mse*I) were confirmed by sequencing multiple clones of the PCR products from random samples (n = 5) (data not shown).

The allele frequencies for the nine loci in the control subjects were fully consistent with Hardy–Weinberg equilibrium (data not shown). There was significant association between atopic asthma and variants of FcεRIβ, HTm4 genes, and D11S480 (Table 1). GIF and D11S1335 centromeric to FcεRIβ (38) showed no association with severe atopy (Table 1). No significant association was found with any form of atopy or asthma phenotype at the other loci.

There was a significant difference in genotype frequencies of FcεRIβ variants between control and atopic asthma subjects (Table 1). This was strongest with the intron 2 variants (OR = 2.98, 95%CI 1.88–4.97, $p < 0.01$). This polymorphism was significantly associated with any combination of atopy phenotypes and showed a highest OR of 4.77 (95%CI 2.45–10.77, $p < 0.01$) with a marked atopy phenotype (high total serum IgE > 300 IU/ml and allergen-specific IgE for HDM and GX both > 3.5 IU/ml of serum). However, the coding variant, Gly237Glu, was not associated with any combination of atopy or asthma phenotype. As shown in Table 1, a genetic association was also found between the variant of HTm4 and atopic asthma (OR = 2.59, 95%CI 1.21–4.88, $p < 0.01$): this variant was associated with marked atopy (OR = 3.31, 95%CI 1.14–13.99, $p < 0.05$), though the highest odds of 6.00 (95%CI 1.45–12.33, $p < 0.05$) was found with HDM > 1.5 IU/ml. In terms of perennial, chronic asthma, the *Rsa*I RFLP in intron 2 of the FcεRIβ gene, as well as *Taq*I RFLP in intron 3 of HTm4 gene showed a slight increase of ORs; 3.37 (95%CI 1.98–8.97, $p < 0.01$) or 3.13 (95%CI 1.41–7.88, $p < 0.01$), respectively (Table 1).

There was a strong linkage disequilibrium between the intron 2 *Rsa*I RFLP and the 3′ tail *Rsa*I polymorphism ($t = \Delta/SE = 2.88$, $p < 0.001$), but this was weaker with the *Mse*I polymorphism in the 5′ promoter region ($t = \Delta/SE = 2.09$, $p = 0.03$) in *FcεRIβ*. The variant of HTm4 gene was in strong linkage disequilibrium ($t = \Delta/SE = 2.44$, $p < 0.001$) with the intron 2 variant of FcεRIβ gene.

One of the alleles, 201 bp of D11S480, was associated with asthma (OR = 2.07, 95%CI 1.00–4.98, $p = 0.05$); OR increased up to 2.77 (95%CI 1.18–7.04, $p < 0.05$) in association with perennial, chronic asthma. However, it did not associate with a marked atopy phenotype (OR = 1.23 95%CI 0.68–5.98, $\chi^2 = p > 0.1$, Table 1) nor any other form of atopy phenotype.

Discussion

In this study, we found two patterns of association with atopic asthma on the 11q13.1 region: in one, FcεRIβ and HTm4 are associated with high serum IgE levels, and in the other, D11S480 is associated with asthma *per se*.

Linkage has been reported between atopic asthma and the FcεRIβ locus in British (1, 2, 39–41), Australian (42), German (4), and Japanese (3, 43) families. A large number of affected sibs from the Australian general population also showed strong linkage between FcεRIβ and asthma, even in the absence of atopy (8). This raises the question whether this linkage is through bronchial hyper-responsiveness or through general IgE responsiveness. In this study, we found strong genetic association between atopic asthma and one of the non-coding variants of the FcεRIβ gene, *Rsa*I RFLP in intron 2. The OR for marked asthma (chronic and perennial) increased to 3.37, whereas the greatest OR was found for marked atopy phenotype. In German families (30), severe atopy was linked to FcεRIβ, though lod scores were negative in tests with mild atopy phenotypes. Multivariate analysis demonstrated that the contribution of FcεRIβ on atopic asthma was stronger

among patients with higher total IgE (Hagihara et al., unpublished data). Furthermore, three coding variants, Leu181Ile, Leu183Val, and Gly237Glu, showed strong association with atopy, especially with high total serum IgE levels in distinct ethnic groups (10–17), while intrinsic asthma did not (17), suggesting that the linkage or association of atopic asthma with FcεRIβ is mainly through IgE responsiveness. This is supported by the association or linkage between FcεRIβ and another clinical atopic disorder, eczema, in Germany (4), Britain (44), and Japan (Ohta et al., submitted), since IgE responsiveness is a common feature between atopic asthma and eczema.

A growing body of evidence supports the candidacy of *FcεRIβ* as an atopy locus. However, the functional action of FcεRIβ variants in promoting atopy remains undefined. A direct functional link of the coding variant, Leu181, in the FcεRIβ gene was hypothesized. However, neither significant upregulation of histamine release from basophils (45) nor of phosphorylation of the receptors has been found (J-P Kinet, personal communication). More recently, the function of human FcεRIβ has been described more precisely; it amplifies the intensity of cell activation signals through the FcεRIγ chain with a gain of 5–7-fold when it couples with FcεRI αγ receptors *in vitro* (46) and *in vivo* (47). This 'amplifier' function is of particular interest, due to several non-coding polymorphisms (16–18), which might relate to expression levels of this gene. Further molecular biochemical studies are needed to test whether these polymorphisms are responsible for quantitative change in FcεRIβ's amplifying signals in basophils and mast cells among atopic subjects.

Another explanation is that an unrecognized atopy gene sits close to *FcεRIβ*, and its variants associate tightly with those of *FcεRIβ*. A new member of the CD20/FcεRIβ family, the HTm4 gene, has been cloned (19), and recent fine mapping enables us to determine the distance between FcεRIβ and HTm4 to less than 70 kb (Adra et al., submitted). HTm4 spans about 13 kb and consists of seven exons, a structure quite similar to that of FcεRIβ (Adra et al., submitted). A *Taq*I RFLP in the third intron of the gene was identified and showed a strong association with atopic asthma. Since this variant showed similar ORs for marked asthma, as well as marked atopy phenotypes, to those for intron 2 of the FcεRIβ gene, *HTm4* might be regarded as a candidate locus for atopy on 11q13.1. Another possibility is a locus between the two genes, *FcεRIβ* and *HTm4*, at which variants confer the atopy phenotype. Further physical and functional mapping is under way in our laboratories.

The second locus for asthma on 11q13.1 is between D11S480 and D11S1883, approximately 5 cM telomeric to FcεRIβ (Fig. 1). We have previously showed strong linkage with clinical symptoms of asthma at D11S480 in 40 British asthmatic families, but not with atopy phenotype (Dubowitz et al., unpublished data). In this study, one of the alleles of D11S480 was associated with marked asthma, but not with any kind of combination of atopy phenotype. These findings suggest that a clinical asthma locus may be localized in close relation to D11S480, independently of the FcεRIβ/HTm4 locus on 11q13.1.

The autosomal recessive disorder, Bardet–Biedl syndrome (BBS), which is characterized by retinal degeneration, polydactyly, obesity, mental retardation, hypogenitalism, renal dysplasia, and short stature, is heterogeneous with at least four loci (BBS1–4) to date (48). Almost half of Caucasian families have been linked to 11q13.1 (BBS1), and the highest lods were found at D11S1883 with no recombination. Interestingly, a quarter of patients linked to BBS1 showed atopic asthma (48). This indicates that BBS1 might be in linkage disequilibrium with an atopic asthma locus between D11S480 and PYGM (Fig. 1). More recently, strong genetic association was found between childhood asthma and CC16 (23), 1 cM centromeric to D11S480 on 11q13.1. Biallelic and microsatellite variants have been identified in this gene. However, no association was found between these variants and asthma phenotype in British (32) and Japanese (49) populations. Another candidate is CHRM1 (33), a muscarinic receptor on airways. However, no association was found in our population. Since our patients show no association with D11S1883, data on this study, on BBS1, and on CC16 suggest that the locus for asthma might be localized between D11S480 and D11S1883 in a 300-kb interval.

A third atopic asthma locus on 11q13.1 has been reported telomeric to FGF3, more than 10–12 cM away from FcεRIβ. In 131 British families, an allelic association was found between D11S527 and bronchial hyper-responsiveness (21). Furthermore, at D11S534, one of the alleles associated with total IgE levels (21). We therefore examined this area with two candidate genes, GSTP1 (36) and UCP2 (39); in the former, variant Ile104Val has been identified and is of particular interest in relation to alcohol- or chemical-induced asthma; the latter is specifically expressed in macrophages. However, no association was found between any kind of atopy or asthma phenotypes and variants of these genes. Australian families linked to FcεRIβ did not favor linkage to loci 8–9 cM either side of this

gene (49, 50). Also, two genome-wide searches (42, 51) failed to find linkage with markers in this area, including FGF3. Since the associations were only found with microsatellite repeats (FGF3, D11S527, and D11S534) in this region and are extremes in a huge number of tests, these associations might be due to a type I error, despite testing in both parametric and non-parametric approaches (21).

In conclusion, there might be two loci for atopic asthma on 11q13.1. One is for atopy (high IgE levels) and lies between D11S1335 and CD20 in an interval of 0.8 Mb, the other is for asthma *per se* (wheeze and labile airway obstruction) and lies close to D11S480. These findings might explain the diversity among the reports, with or without association and/or linkage to different loci on 11q13.

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Clinical status and optimal use of rituximab for B-cell lymphomas.

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Rituximab (IDEC-C2B8 [Rituxan]) is a chimeric anti-CD20 monoclonal antibody (MoAb) that was recently approved by the FDA for the treatment of patients with low-grade or follicular B-cell non-Hodgkin's lymphoma. Its potential efficacy in other B-cell malignancies is currently being explored. This article reviews the mechanisms of action of rituximab, as well as preclinical data and results of the clinical trials that led to its approval. Also discussed are the mechanics of administering rituximab on the recommended weekly x 4 outpatient schedule. Finally, the article describes ongoing and planned trials of rituximab in other dosage schedules, in other B-cell neoplasms, and in conjunction with chemotherapy. As the first MoAb to gain FDA approval for the treatment of a malignancy, rituximab signals the beginning of a promising new era in cancer therapy.

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